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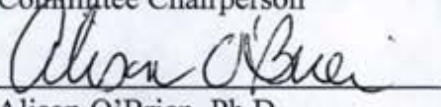
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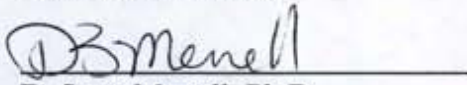
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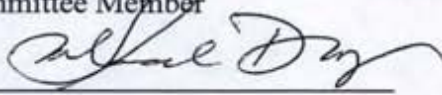
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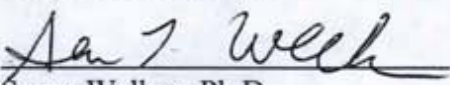
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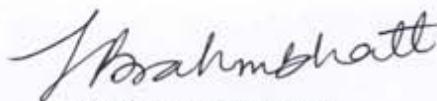
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Abstract

Title of Dissertation:

Bacillus Collagen Like Protein of Anthracis: Immunological and Functional Analyses

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Thesis directed by:

Alison O'Brien, Ph.D.

Professor and Chair, Department of Microbiology and Immunology

Interest in the development of an improved anthrax vaccine has increased since the 2001 bioterrorist event that involved the public dispersal of *Bacillus anthracis* spores through the United States postal system. The currently licensed anthrax vaccine adsorbed (AVA) is a crude extract in which the primary protective immunogen is the toxin binding component of the anthrax toxins, called protective antigen (PA); this antigen is given to elicit toxin-neutralizing antibodies in the recipient. Unfortunately, AVA has several

drawbacks not the least of which are that it is administered as a six dose series over an extended period and that painful local reactions to the vaccine are commonly reported. Moreover, AVA may be less than optimally effective against the most lethal form of the disease, inhalational anthrax. Therefore, the first goal of this study was to explore the use of a *B. anthracis* spore surface antigen as a means of increasing protection afforded by a Protective Antigen (PA) - based vaccine.

Bacillus collagen-like protein of anthracis (BclA) is an immunodominant glycoprotein located on the exosporium of *Bacillus anthracis*. We hypothesized that antibodies to this spore-surface antigen are largely responsible for the augmented immunity to anthrax that has been reported for animals vaccinated with inactivated spores and Protective Antigen (PA) compared to PA alone. Here, the capacity of recombinant, histidine-tagged, non-glycosylated BclA (rBclA) given with adjuvant, with and without PA, to protect A/J mice against ten 50% lethal doses of Sterne strain spores introduced subcutaneously was evaluated. I also evaluated the capacity of rabbit anti-rBclA IgG administered intraperitoneally to mice before spore inoculation to afford protection. Additionally, anti-rBclA IgG was evaluated for its opsonophagocytic and germination retardation properties. Since BclA has some sequence similarity to human collagen, I also tested the extent of binding of anti- rBclA antibodies to human collagen types I, III and V and found no discernable cross reactivity. Taken together, these results support the concept of rBclA as a safe and effective boost for a PA-primed individual against anthrax and further suggest that such rBclA- enhanced protection occurs by induction of spore-opsonizing and germination-inhibiting antibodies.

The second objective of this project was to evaluate the role of BclA in spore biology. Specifically, I sought to assess the impact of BclA on three characteristics of the spores: germination, surface charge, and interaction with host extracellular matrix proteins. For these purposes, we constructed a marker-less *bclA* null mutant in *B. anthracis* Sterne 34F2 strain. The growth and sporulation rates of the Δ*bclA* and parent strains were nearly indistinguishable, but germination of mutant spores occurred more rapidly than wild-type spores *in vitro* and was more complete by 60 minutes. Additionally, the mean time-to-death of A/J mice inoculated subcutaneously or intranasally with mutant spores was lower than for the wild-type spores, a finding that suggests a more rapid germination rate of the mutant than wild type spores *in vivo* at that dose. However, the 50% lethal doses of the two strains were similar. I speculated that these *in vitro* and *in vivo* differences between mutant and wild-type spores might reflect ease of access of germinants to their receptors in the absence of BclA. I also compared the hydrophobic and adhesive properties of Δ*bclA* and wild-type spores. The Δ*bclA* spores were markedly less water-repellent than wild type spores and, probably as a consequence, the extracellular matrix proteins laminin and fibronectin bound significantly better to mutant than to wild type spores. From these results we speculate that BclA acts as a shield to not only reduce the ease with which spores germinate but also to change the surface properties of the spore which, in turn, may impede the interaction of the spore with host matrix substances.

Bacillus Collagen Like Protein of Anthracis:

Immunological and Functional Analyses

By

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To my wonderful mom:

For showing me what strength can accomplish

To my beautiful children: Rehani, Aarohi and Pratosh:

Your faith in mommy power gives me the strength

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Chapter One

Introduction

Preface

Bacillus anthracis, one of 70 species within the genus *Bacillus,* is a spore-forming, Gram-positive, non-motile bacterium that is the causative agent of anthrax. Spores, the infectious form of anthrax can persist in the soil in a dormant stage for decades. Herbivores are the primary reservoirs of anthrax while humans are only coincidental hosts. The Centers for Disease Control and Prevention has labeled *B. anthracis* a category A infectious agent. Organisms so classified are highly virulent for humans and are either easily disseminated and/or are readily transmitted from person to person. In the case of *B. anthracis*, its spores are relatively simple to prepare and disperse. However, the disease is not spread from person to person.

Anthrax can be contracted by individuals who have been exposed to spores cutaneously, by ingestion, or by inhalational. When acquired by the inhalational route, anthrax can prove highly lethal with mortality rates reaching as high as 90%. The chances of a *B anthracis*-infected person surviving can be markedly improved by early diagnosis, aggressive intensive care, and long term combination antibiotic therapy. On the prevention side, the current FDA approved vaccine faces challenges such as limited availability, reactogenicity and a requirement of multiple doses. The present focus of research in the vaccine arena is on exploration of additional antigens, such as spore proteins, to increase efficacy of a Protective Antigen (PA) based vaccine.

The following introduction to this thesis is divided into several sections. First, an overview of *B. anthracis* is provided. The content of this segment includes brief synopses of anthrax disease history and epidemiology, use of *B. anthracis* spores as an

agent of bioterrorism, pathogenic determinants of *B. anthracis*, anthrax disease pathology, and the current state of anthrax treatment and prevention. Second, the major immunogenic exosporium protein, BclA is described. Third, the findings on the hydrophobic nature of spores and the potential role of the exosporium in this water-repellent feature of spores are reviewed. Finally, in the last part of the introduction, the hypothesis and specific aims of this dissertation are presented.

History

A "plague of boils" described in the book of Exodus (9:8-9) of the Old Testament, is considered by many to refer to anthrax, "And it will become fine dust over all the land of Egypt, and will become boils breaking out with sores on man and beast through all the land of Egypt." Anthrax was subsequently blamed for livestock losses in the 1600s and 1700s and there were some accounts of human cases in about the same period (3, 4). In the early-1800s, a human case in Kentucky was reported of a man who contracted anthrax after contact with infected livestock. Human cases of cutaneous anthrax were also documented in England. The disease is known as "ragpicker's disease" or "woolsorter's disease because it occupationally affected workers in those trades. In the mid-1800s the causative agent of anthrax was first viewed under a microscope. Casimir Davaine and Pierre Rayer first observed rod-like organisms present in the blood of anthrax-infected animals and humans in 1850. By 1863, Davaine showed that those rods were most likely the cause of anthrax since unexposed sheep did not develop the disease. About 20 years later, in 1877, Robert Koch correlated infection with what is now known as *Bacillus anthracis* with the disease anthrax. Around this same time, Louis Pasteur became interested in the disease because of a devastating outbreak of anthrax among sheep. He was the first researcher to begin to develop an anthrax vaccine. However, another scientist, W.S. Greenfield, did similar research and used *B. anthracis* cultures grown at high temperature as a vaccine to produce immunity in cattle (114). Greenfield's work actually preceded Pasteur's, but because Greenfield was less well known than Pasteur, Pasteur got most of the credit for the discovery.

Organism and epidemiology

Anthrax is caused by the bacterium *Bacillus anthracis*. *B. anthracis* is derived from the Greek word, anthrakis, which translates into "coal" because the organism causes dark, coal-like eschars on affected areas (58). *B. anthracis* is an aerobic, gram-positive, non-motile rod (70). The bacterium measures 1-1.5mm by 3-10mm (57). The vegetative cell forms spores upon sporulation, a process triggered by unfavorable conditions like nutritional deprivation or exposure to air (Fig.1). Spores are metabolically dormant and are resistant to many environmental factors such as heat, radiation, desiccation, pH extremes, and toxic chemicals (87). Cultivation of *B. anthracis* can be accomplished on non-selective microbiological media at 37°C (58). The colonies are 4-5 mm in diameter, rough, off-white in color with a characteristic sandy or ground glass appearance with comma shaped projections giving it the name "medusa head" (57, 58). *B. anthracis* occurs singly or in pairs in tissues and forms long chains in culture giving it the "boxcar" appearance (40). *B. anthracis* belongs to the *B. cereus* group of bacilli which includes *B. cereus, B. thuringiensis*, and *B. mycoides (35).* Anthrax can be differentiated from other members of the group by several methods. All members of the *B. cereus* group except *B. anthracis* are resistant to penicillin because of a chromosomally encoded betalactamase (35). Other characteristics include the absence of hemolysis, lack of motility and the presence of an antiphagocytic capsule consisting of D-glutamic acid (57).

Anthrax is most common in South and Central America, southern and Eastern Europe, Asia, Africa, the Caribbean, and the Middle East (http://www.bt.cdc.gov/agent/anthrax/basics/factsheets.asp). Natural human infection is

usually acquired following contact with infected animals or contaminated animal products (2) There are no known cases of human-to-human transmission (35). The natural form of anthrax is extremely rare in the United States; only 244 cases were reported between 1944 and 1999. A small number of anthrax cases linked to consumption of undercooked contaminated meat have also been reported (35). Anthrax is most often found in herbivore animals that are infected by ingestion of spores from the soil (2) Studies of agricultural outbreaks suggest that anthrax bacilli favor soil with a pH > 6.0 and one that is rich in organic matter. Major changes in the soil microenvironment, such as drought or rainfall, enhance sporulation (104).

Figure 1

Sporulation and germination cycle of *B. anthracis*

A vegetative cell experiencing a stress condition, e.g., nutritional deprivation, undergoes asymmetrical cell division. The mother cell lyses and produces metabolically inert but infectious spore. Germination is triggered by the presence of nutritional stimulants. A spore hydrates and grows out into a vegetative form that is capable of toxin production.

1. Starvation
2. Asymmetrical division
3. Mother cell lysis
4. Germination
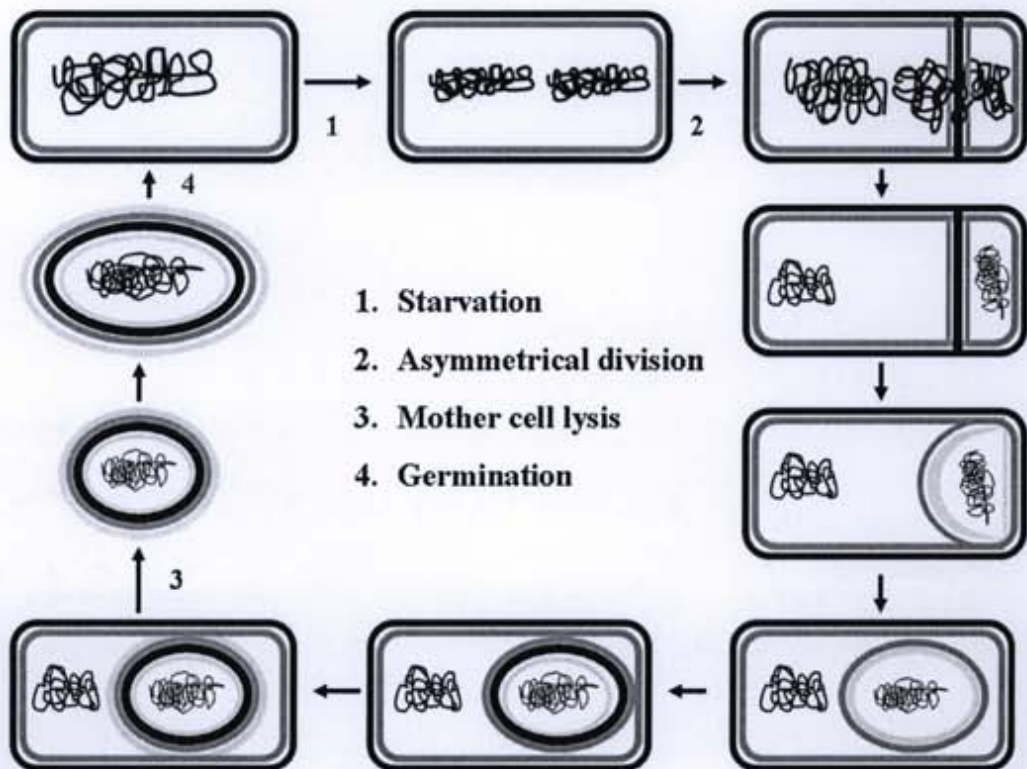
Anthrax as a bioterrorism agent

Research on the use of anthrax as a weapon dates back about 80 years (83). Today, at least 17 nations are believed to have offensive biological weapons programs; however, it is uncertain how many of these countries are working with anthrax. Inhalation anthrax is the method of infection associated with biological warfare. Anthrax biowarfare and bioterrorism concerns are based on the fact that infective spores can be made and purified in large quantities and in a non-aggregable powder form ideal for aerosol dissemination. Another characteristic that makes anthrax spores an effective biological agent is that these spores can be stored for decades without losing viability (32). The small quantity of anthrax needed for a lethal inhalation dose makes concealment, transportation, and dissemination very easy. An anthrax aerosol is odorless and invisible, characteristic that make such a cloud a potentially stealthy killer. Moreover, only one millionth of a gram of anthrax is a lethal dose. A kilogram could prove lethal to hundreds of thousands of individuals living in a metropolitan area.

The first recorded use of anthrax as a weapon was in 1915. German agents in the U.S. injected horses, mules and cattle being shipped to Europe with the anthrax bacterium. In 1937, Japan began a biological warfare program in Manchuria that included tests involving anthrax. In 1942, the United Kingdom experimented with methods to distribute anthrax spores off the coast of Scotland on Gruinard Island. These spores persisted and remained theoretically capable of infection for decades after dispersal. A subsequent decontamination effort, took nearly 10 years to clean up the island. The

United States began developing anthrax as a biological weapon in 1943. In 1969, President Nixon declared an end to the U.S. offensive biological weapons program but maintained support for an operative defensive program. In 1970, a World Health Organization (WHO) expert committee estimated that there could be 100,000 casualties following a theoretical aircraft release of 50 kg of anthrax over a developed urban population of 5 million [WHO 1970]. In 1972, the development and stockpiling of biological weapons was outlawed by the International Biological Weapons Convention. Nevertheless, aerosolized anthrax spores were accidentally released in May 1979 at a Soviet Union military research facility in Sverdlovsk, an event that resulted in one of the most deadly anthrax epidemics known. There were 96 victims in all; seventeen had skin infections and survived, seventy-nine had intestinal infection, and, of these, 64 died. This outbreak demonstrated the potentially deadly power of an anthrax aerosol (84).

The terrorist group Aum Shinrikyo, known for their release of sarin, a type of nerve gas, in a Tokyo, Japan subway station in 1995, dispersed aerosols of anthrax throughout Tokyo on at least 8 occasions. For reason that are not clear, these incidents did not lead to any reported illnesses. In 1995, Iraq admitted to producing 8,500 liters of concentrated anthrax spores as part of biological weapons program. Shortly after the attacks of September 11, a series of letters were mailed anonymously to several news media companies and government officials in the United States. The letters contained a high-grade, finely textured version of anthrax later identified as identical to the virulent Ames strain, an isolate that was acquired by the U.S. Army in the early 1980s. During this bioterrorism event, 22 individuals were infected by the anthrax bacterium and, of

these, five people died from inhalation anthrax. These series of events confirm the clear

and present threat of the use of anthrax spores as a bioterrorism agent.

Pathogenic determinants

Full virulence of *B. anthracis* for most mammals requires expression by

vegetative cells of both an anti-phagocytic poly-D-glutamic acid capsule (48, 80, 119)

and anthrax lethal and edema toxins (78) Each of these toxins is a binary molecule

comprised of an enzymatic domain [lethal factor (LF) or edema actor (EF)] and a

receptor- binding component, PA, that is shared by the toxins. PA is also required for

assembly, internalization, and delivery of lethal toxin and edema toxin to the target-cell

cytosol. The secreted binary toxins of *B. anthracis* are responsible for cell killing and

host death (27, 90)

The genes that encode PA, LF, and EF (*pagA, cya, and lef*) are carried on a self-

replicating 184-kb plasmid called pXO1. A second plasmid, the 90-kb pXO2, encodes

genes for formation of the capsule; this plasmid is absent in Sterne and Sterne-like strains

that have been used as live vaccines in livestock (116)[and see *"Vaccines"* section

below]. The virulence of the Sterne strain for A/J and some other strains of mice is due to

toxin production (126) and infection at high doses provokes the two principal toxin-

related characteristic symptoms of anthrax: edema and shock-like death. Antibodies to

the capsule, PA, LF, and EF can be found in the sera of patients recovering from

cutaneous anthrax (107), a finding that suggests that immune responses to the virulence determinants are important in host protection against anthrax.

PA is so named because it can induce antibodies that protect against intoxication of lethal toxin (LeTx) and edema toxin (EdTx) by preventing their binding to PA and can thus confer immunity to spore challenge. This cell binding subunit is a 735 aminoacid protein (M.W. 82,684 kDa), also known as PA83. After PA binds to a cell surface receptor, it is cleaved by a furin-like protease (68), to yield a 63 kDa fragment (PA63) and an N-terminal 20 kDa fragment (PA20). Cleavage is essential for toxin action. The PA 63 peptide assembles into a ring-shaped heptamer structure in solution when bound to its cellular receptor, as was first shown by electron microscopy of purified PA63 (85). The assembly of PA63 into a heptamer exposes the binding site for EF and LF. The heptameric PA then engages LF or EF, enzymatic subunits that can compete with each other for association with PA. Two separate sites on PA may be involved in the binding of LF and EF, as revealed by monoclonal antibody studies. The current data support the model that each PA63 monomer binds one EF or LF molecule with a projected seven molecules of LF and/or EF on a heptamer of PA (106). Once assembled, the toxin complex is internalized by receptor-mediated endocytosis (6).

PA binds to ubiquitously expressed cellular receptors that include tumor endothelium marker 8 (TEM8) and human capillary morphogenesis protein 2 (CMG2), also called anthrax toxin receptor 1 (ATXR1), and anthrax toxin receptor 2 (ATXR2). Engagement of these different receptors by PA may lead to variation in intoxication by EdTx and LeTx (20, 102, 103). EdTx and LeTx require passage through an acidic vesicle. At acidic pH in the endosome, PA heptamers undergo conformational changes and

convert from prepore to pore, and this transition is followed by the translocation of LF/EF into the cytoplasm. EF and LF participate actively in their translocation, since they interact with lipid bilayers in a pH-dependent manner (69). These interactions are optimal at acidic pH and are irreversible for EF but reversible for LF. LF is completely translocated into the cytoplasm, whereas EF remains membrane associated (51)

LF is a 776 amino acid zincdependent metalloprotease (85 kDa) that cleaves the N-termini of several intracellular mitogen-activated protein kinase kinase (MAPPK) members (27, 90). Cleavage of MAPKK blocks several signal transduction pathways that include ERK (extracellular signal-regulated kinase), p38, and JNK (c- Jun N-terminal kinase) pathways (38, 39, 121). EF is a 767 amino acid Ca^{2+}- and calmodulin- dependent adenylyl cyclase that catalyses the conversion of ATP into cAMP (45, 89, 108), an enzymatic activity that alters electrolyte fluxes, affects water homeostasis, and causes edema (76, 77). EF is also reported to impair neutrophil function (30, 88, 122).

Disease pathology

Anthrax is classified as a disease by the route of exposure to spores, since infection by contact, inhalation, or ingestion may lead to different clinical presentations. Regardless of the route of infection, the pathology of anthrax is primarily mediated by expression of LeTx and EdTx, the antiphagocytic function of the *B. anthracis* capsule is also essential for the full spectrum of anthrax disease.

Cutaneous anthrax

The majority of naturally-acquired anthrax in humans is of the cutaneous form. Disease occurs after spores are inoculated through a break or cut in the skin (35, 104). Cutaneous anthrax, as mentioned previously, is usually acquired through contact with infected animals or animal products. Incubation is typically 2–3 days, although first clinical symptoms can appear as early as 12 hours and as late as 2 weeks after the exposure (35, 57). Cutaneous anthrax begins as a pruritic papule that becomes vesicular and then necrotic. This skin lesion is often accompanied by significant edema (35, 57) an observation consistent with the expression of EdTx at the site of infection (35, 57). A characteristic black edematous lesion (eschar) that is usually painless develops and usually clears in 1 to 2 weeks (58). Most cases of cutaneous anthrax resolve spontaneously, although mortality can be as high as 20% in cases not treated with antibiotics (58). The histology of cutaneous anthrax reveals necrosis, lymphocytic infiltration, and edema; in contrast, the pathology associated with suppurative infections is characterized by infiltration of polymorphonuclear leukocytes.

Inhalational anthrax

The most deadly form of anthrax is pulmonary or inhalational anthrax that results from the inhalation of airborne spores (18). Inhalational anthrax is characterized by the rapid progression of flu-like symptoms to overwhelming septicemia and massive organ failure. Over half of the confirmed anthrax cases in the 2001 bioterrorism incident were inhalational anthrax, and these infections resulted in over 50% mortality, even with supportive care and aggressive antibiotic therapy (65). Spores may remain dormant in the lung for extended periods. The initial clinical symptoms are not distinctive: fever, cough, and malaise. This non-specific presentation progresses to significant dyspnea and

cyanosis but without pulmonary infiltrates in most cases (18). Instead, anthrax patients characteristically develop mediastinitis (observed on chest films as a widened mediastinum) and marked pleural effusions. Ultimately, many inhalational anthrax patients display septic shock, fall into a coma, and die.

Such symptoms as described above have also been observed in experimentally-infected primates. Human data have been obtained from studies on victims of an accidental release of anthrax spores from a former Soviet biological warfare facility in Sverdlovsk in 1979. In that incident, anthrax disease appeared anywhere from 2 to 43 days after exposure of the affected individuals to the spores (84). Extended onset of disease after infection was also observed during experimental aerosol inoculation of monkeys (62). In the 2001 bioterrorism event, no cases were observed that could have been classified as extended; rather, the average incubation period was approximately 4 days (65). Because viable spores can be demonstrated in the mediastinal lymph nodes of monkeys long after primary exposure, and because of the possibility of prolonged residence of spores in the lung, primary antibiotic treatment lasting for 60 days is justified and was used in the 2001 incidents. The likelihood of persistent residence of spores *in vivo* also justifies the use of the anthrax vaccine as a post-exposure prophylactic—in conjunction with antibiotic therapy. The human LD_{50} is impossible to know but has been extrapolated from nonhuman primate data to be 4000 to 8000 or as many as 60,000 (62, 120) spores derived from a virulent strain. These data imply that a few spores may be capable of causing infection in a small percentage of a large population due to a combination of host susceptibility factors, lung pathology, and variations in innate virulence of the aerosolized spores.

The current model of the progression of inhalational anthrax infection is as follows. Alveolar macrophages ingest spores that have been deposited in the lung. These cells then migrate via the lymphatics to mediastinal lymph nodes where spore germination occurs (45, 120). Spores survive phagocytosis by macrophages and germinate within phagosomes (34, 49, 50). Germinated spores lyse the host cells, and vegetative bacteria escape to the general circulation and cause septicemia and toxemia through elaboration of LeTx and EdTx.

Gastrointestinal anthrax

Gastrointestinal anthrax is the rarest form of natural anthrax and is thought to result from ingestion of insufficiently cooked contaminated meat. Two distinct syndromes have been observed: oral-pharyngeal and abdominal. Little information is available about the risks of direct contamination of food or water with *B. anthracis* spores. However, gastrointestinal infection could occur after consumption of large numbers of vegetative cells, such as those found in raw or undercooked meat from an infected herbivore (Fig. 2).

Figure 2

Transmission cycle of *B. anthracis*

The natural transmission cycle of anthrax involves herbivores. The herbivores come in contact with *B. anthracis* spores and develop the disease. When the infected animal dies, the vegetative bacilli sporulate, and the spores contaminate the environment. The spores infect other healthy animals and the cycle continues. Humans are coincidental hosts and generally contract the disease by coming in contact with infected animal products such as meat, wool, or hides.
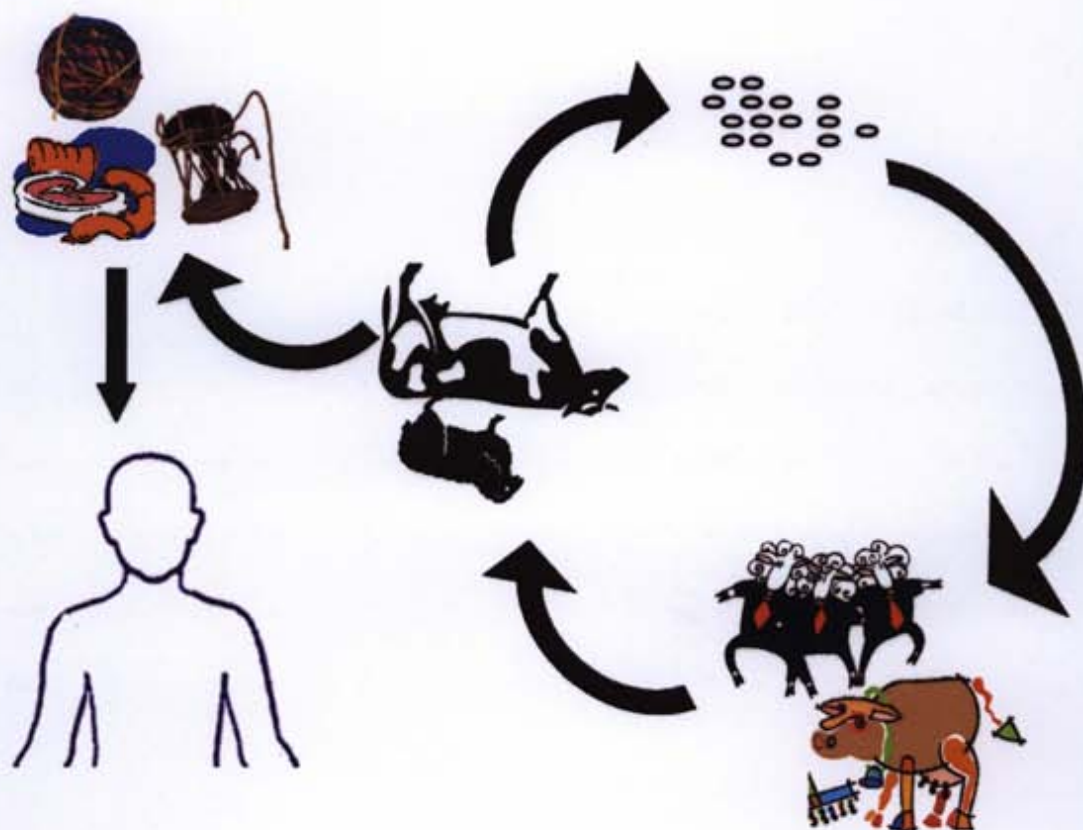
Vaccines

Protection against anthrax, as conferred by immunization with the spores of *B. anthracis,* was first described (Greenfield in 1880, and Pasteur in 1881) over 120 years ago. The attenuated *B. anthracis* strain, isolated by Pasteur, was used as the first live bacterial vaccine (25). Initially, the vaccine schedule of Pasteur was adopted (two doses of partially heat-inactivated spores), but variability in potency and virulence prompted a search for a more effective and stable vaccine (118). The success of the attenuated Sterne veterinary vaccine in the 1930 presaged a global reduction of anthrax cases in livestock in response to national programs (117). This decrease in animal cases led to a subsequent decrease in human cases. Unfortunately, there are still regions in the world where anthrax is endemic. This endemicity generally reflects the quality of public health and veterinary services in the respective regions (56). At present, the most widely used vaccine for the prevention of anthrax in animals is the live Sterne strain vaccine. This vaccine is based on a toxigenic, live attenuated variant of *B. anthracis* developed by Sterne in 1937 (strain 34F2). This non-encapsulated strain lacks plasmid pOX2 that encodes for capsule formation. In Russia and China, live spore vaccines equivalent to the Sterne strain are in use (strain 55) in humans, while in Italy animals are vaccinated with a strain (Carbosap) that still carries both virulence plasmids, but is attenuated (8). After a single subcutaneous vaccination [5000–10,000 live spores per dose depending on type of vaccinated animal in 0.1% saponin], immunity develops within 7–10 days. While a single dose of Sterne strain spores will provide immunity for about a year, repeated vaccinations (six doses) are required for long-term protection (118). The residual virulence of all these

live vaccine strains, i.e., local side reactions and occasional death discouraged their use in humans (117).

To overcome safety problems of spore-based vaccines for humans, acellular vaccines have been developed. The primary rationale for such improved vaccines has been to counter threats of biological warfare or bioterrorism that have occurred since the end of World War II. In the US, a cell-free culture filtrate adsorbed onto aluminium hydroxide from non-encapsulated non-proteolytic strain V770-NP-R is used for human vaccination. The currently licensed U.S. vaccine for human use is called Anthrax vaccine adsorbed (AVA) or Biothrax and is manufactured by BioPort Corporation, MI. This vaccine was licensed by the FDA in 1970 based on safety studies conducted in 7000 participants who received 16,000 doses of AVA (94, 95, 105). The principal antigenic component of AVA is PA with smaller amounts of LF and EF. The vaccine composition varies from lot to lot and is not fully characterized. The UK vaccine is an alum precipitate of cell-free culture supernatant of the non-encapsulated toxigenic strain 34F2 (Sterne) (117). Both vaccines are licensed to be given in six initial doses at 0, 2, and 4 weeks and 6, 12, and 18 months followed by a yearly booster [CDC].

Based on review of cumulative epidemiological information, the Institute of Medicine (IOM) concluded that AVA is acceptably safe and is not associated with more frequent local or systemic side effects than numerous other vaccines (1). Despite the conclusion that AVA is safe and effective, the residual concerns over long-term and short-term health effects of AVA persist. AVA has additional shortcomings that warrant the development of replacement vaccination strategies. AVA is based on a manufacturing process that does not currently control for the content of the PA or other elements, such

as residual EF and LF; these other components probably contribute to both protective immunity and local and systemic reactions. The licensed schedule of immunization to achieve protection with AVA is based on a 6-dose series, an inoculation regimen that is based on historical rather than immunological rationale. For an improved AVA vaccine, the immunization timetable could be altered. Indeed, two doses of AVA given in a biweekly interval induced antibodies in 96% of human subjects with less local reactivity than the licensed regimen (94, 95). Anti-PA titers were suboptimal in comparison to three doses given at a biweekly interval. However, 2 doses, given 4 weeks apart induced antibodies equivalent to the 3 dose series during the same time period.

Current research efforts aimed to develop better or alternative vaccines, include: production of purified antigenic polypeptides, attenuated strains, recombinant vaccine vectors, and DNA vaccines (13, 22, 129). The identification of immunological correlates of protection in the case of anthrax vaccines is difficult to obtain due to the low incidence of disease, and this issue hampers vaccine efficacy studies. All data reported to date underscore that the titer of anti- PA antibody is predictor of survival and could be used as surrogate marker for protection (19, 44, 93, 100, 117, 124). Nevertheless, spore vaccines appear to be more efficacious than vegetative cell- vaccines or recombinant PA (rPA) vaccines, indicating that spore antigens may contribute to protection (21, 26, 79, 91, 92, 125). One such spore protein is Bacillus Collagen Like Protein of anthracis (BclA), the focus of this study.

Treatment

In vitro B anthracis is susceptible to penicillins, fluoroquinolones, tetracycline, chloramphenicol, aminoglycosides, macrolides, imipenem/meropenem, rifampicin, and vancomycin but resistant to cephalosporins, trimethoprim, and sulfomanides. Penicillin has long been considered the drug of choice and only rarely has penicillin resistance been found in naturally occurring strains.

Table 1 shows the current guidelines for the recommended treatment of inhalation anthrax, by the Center for Disease Control. Because the Ames strain that caused the recent infections in the USA contains inducible B lactamases, the treatment of systemic anthrax with penicillin or amoxicillin alone is not now recommended. For mild cases of cutaneous anthrax, treatment with ciprofloxacillin (500 mg twice daily), doxycycline (100 mg twice daily), or amoxicillin (500 mg three times daily) is recommended. In the context of a bioterrorist attack, treatment should continue for 60 days as opposed to seven to 10 days for naturally acquired disease.

Post-exposure prophylaxis is not suggested for asymptomatic people, unless public health or other medical authorities deem that they have been exposed to a credible threat of anthrax spores. A long period (60 days) of prophylaxis is recommended because of the prolonged latency period that can elapse before germination of the inhaled spores occurs. Ciprofloxacin is currently considered the prophylaxis of choice and table 2 lists current treatment guidelines.

Table 1

Treatment recommendations for inhalation anthrax

Patient category	Intravenous (IV) treatment	Long term treatment‡
Adult*	Ciprofloxacin 400 mg IV bd or doxycycline 100 mg IV bd plus 1 or 2 other antibiotics†	Switch to oral treatment when clinically appropriate. Ciprofloxacin 500 mg bd or doxycycline 100 mg bd to complete 60 days
Children	Ciprofloxacin 10–15 mg IV bd. Doxycycline >8 years >45 kg: 100 mg IV bd 8 years <45 kg or <8 years: 2.2 mg/kg bd +1 or 2 other antibiotics†	Switch to oral antibiotic when clinically appropriate. Ciprofloxacin 10–15 mg/kg bd or doxycycline (same dose regimen) to complete 60 days

Once patients have stabilized clinically, the IV treatment may be switched to oral and monotherapy may be used to complete the 60 day course. Other antibiotics that are active in vitro against the current strain are: ampicillin, penicillin, clindamycin, clarithromycin, imipenem/meropenem, vancomycin, rifampicin, and chloramphenicol.

*Pregnant women and immunocompromised patients should receive the same treatment; †consider steroids with severe edema or meningitis; ‡one drug may be used when the Patient has stabilized. bd,twice daily (5).

Table 2

Recommended prophylaxis after exposure to *Bacillus anthracis*

Antimicrobial agent	Adults	Children

Oral fluoroquinolones
 	
Ciprofloxacin
	500 mg bd	20–30 mg/kg of body mass
		
		daily, divided into doses—
		
		as a guide
		
		  10 kg: 125 mg bd
		
		  20 kg: 250 mg bd
		
		  30 kg: 375 mg bd
		
		  40 kg: as for adult

If fluoroquinolones are not available or are contraindicated Doxycycline	100 mg bd	5 mg/kg body mass/day divided into two doses

Ciprofloxacin is not licensed for use in children or pregnant women. There have been no formal studies of the use of ciprofloxacin during pregnancy, but it is unlikely to be associated with a high risk of abnormalities of fetal development. There is some evidence that the use of fluoroquinolones in children (including use by breast feeding mothers) may be associated with tendinopathy and arthropathy. If *B. anthracis* exposure is confirmed, the organism must be tested for penicillin susceptibility. If susceptible, exposed persons may be treated with oral amoxicillin as an alternative to ciprofloxacin or doxycycline (40 mg/kg of body mass/day in divided doses 8 hourly; not to exceed 500 mg, three times daily). Pharmacokinetic studies have shown that ciprofloxacin achieves

far higher concentrations in lung macrophages than does penicillin, and is therefore a more effective prophylactic antibiotic. The risk of adverse effects must be weighed against the risk of developing a serious disease. Ciprofloxacin has the added advantage that it is also an effective prophylactic treatment for other potential agents that may be used in deliberate release scenarios, such as plague and tularaemia.

bd, twice daily (5)

BclA

The spores of *B. anthracis* are covered by a loose balloon-like structure called the exosporium (47). Electron microscopy revealed the exosporium to be a paracrystlline basal layer with hair-like projections (14, 47, 52, 72). The exosporium consists of proteins, lipids and carbohydrates (14, 81, 82). Of the exosporium proteins that have been described (14, 23) several are glycoproteins. Exosporium- specific glycoproteins have also been found in species that are closely related to *B. anthracis*, i.e., *B. thuringiensis* (46) and *B. cereus* (24).

Bacillus collagen like protein of anthracis (BclA) was first described in 2002 by Sylvestre et al., and, is detectable 4 hours after the vegetative cell enters into sporulation (111). BclA is a 382 is amino acid glycosylated protein that has several unique features (111) . The N-terminus of BclA is anchored into the basal layer of the exosporium, and the C-terminus is exposed on the outside (15). The middle region of BclA, termed collagen like region (CLR), is comprised of 70 triplet repeats (GXX) that include 54 GPT triplets. Sylvestre et al. also reported that polymorphism in the CLR leads to variations in the filament length of BclA (112) (fig. 3). Nearly all the BclA triplet repeats contain a threonine residue that provides potential sites for O-glycosylation. In fact, two O-linked oligosaccharides, a 715-Da tetrasaccharide and a 324-Da disaccharide are believed to be attached to BclA through a GalNAc linker (33). Multiple copies of the tetrasaccharide are linked in the CLR, whereas the disaccharide is proposed to be attached outside the CLR. The structure of the tetrasaccharide is 2-*O*-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-L-rhamnopyranose (33). There is a non- reducing terminal sugar on this

tetrasaccharide called anthrose (33). Anthrose is unique to *B. anthracis* and not found in

the closely related *B. thuringiensis* or *B. cereus* species. Two exosporium proteins,

ExsFA and ExsFB, are required for the proper localization of BclA on the surface of the

spore. The function of BclA is unknown, but Sylvestre et al. (111) and Boydson et al.

(15) suggested that it may play a role in initial interaction with the environment and/or

host.

Figure 3

Schematic structure of BclA

A schematic diagram of the *B. anthracis* spore with its hair or filament-like appendages made of BclA.

The Sterne BclA protein is 400 amino acids long. The mature protein starts at 20 amino acids. The amino terminus, or spore anchoring end, is comprised of 21-40 amino acids. The central region of 70 GXX motifs includes 54 GPT repeats of the collagen like region. The carboxy terminus consists of amino acids 251-400.

Structure of BclA

20 41   I = GPT ☐ = [(GPT)₅GDTGTT]₂ 250   400

H_2N- ... -COOH
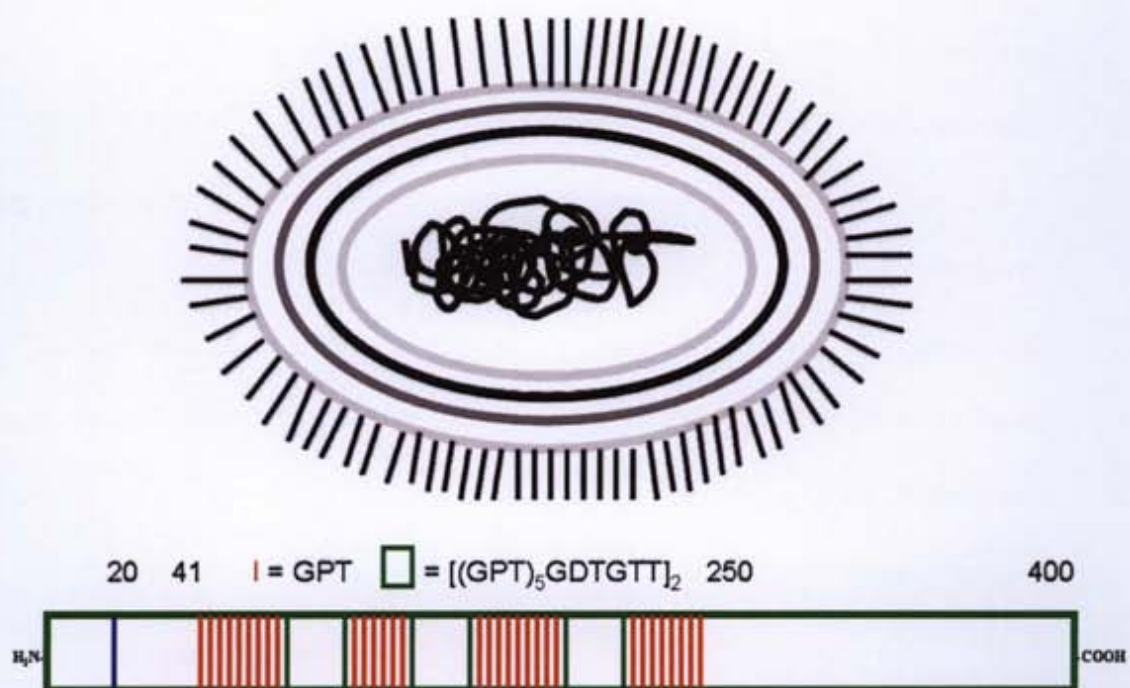
Hydrophobicity

Hydrophobic interactions function in adherence of microorganisms to a wide variety of surfaces, both animate and inanimate. The hydrophobic nature of the outermost surface of microbes has been cited as a factor in the partitioning of organisms at interfaces, in the adherence of bacteria to nonwettable plastic surfaces, and in the attachment of bacteria to phagocytes and other mammalian cells. Craven and Blankenship demonstrated the hydrophobiciy of *C. perfringens* spores and noted that the heat treatment that promotes more rapid spore germination increases the hydrophobicity of those spores (29). Spores of *B. megaterium* strain QM B1551 that have an exosporium were demonstrated to be more hydrophobic than those lacking an exosporium (71, 113). An association between spore hydrophobicity and the presence of an exosporium was also reported for several other *Bacillus* species. Kutima and Foegeding (73) reported a decrease in hydrophobicity of the spores of *B. cereus* strain T when spore coats were removed by chemical treatment. Weineck et al. (128) also reported decreased adherence to hexadecane of an outer coat negative spore mutant of *B. megaterium* strain ATCC 33729 compared with wildtype strain *B. megaterium* strain ATCC 12872 This later finding suggests that the outer spore coat or exosporium plays a role in the hydrophobicity of the spore. Both Weineck et al. and Doyle et al (37, 128) also found that heat treatment increased spore hydrophobicity. Doyle et al. suggested that the increases in the spore hydrophobicity after heat treatment may result from the disruption of outer coat or exosporium proteins. Alizadeh et al reported that increased temperature can alter the structure of macromolecules cause an unfolding of the proteins and expose internal

hydrophobic moieties (9). Additionally, Doyle et al. (37) suggested that hydrophobic interactions are important in the attachment of the spores to environmental proteins. Thus, increased spore hydrophobicity because of heat activation may increase the affinity of the spore for proteins or lipids and thus provide a nutritional source for outgrowth of the vegetative cells following spore germination. Even though spore hydrophobicity has been attributed to the presence of an exosporium, the specific constituents of exosporium responsible for the hydrophobic nature of the spores have been not identified.

SPECIFIC AIMS OF THIS DISSERTATION

The goals of this dissertation are to investigate the efficacy of BclA as a spore antigen and as a vaccine candidate and to study its function. The hypothesis of this work is given below.

Antibodies to the *B. anthracis* exosporium protein BclA may contribute to the protective immune response.

The specific aims of this dissertation are as follows:

1. Clone, express and purify BclA and assess its immunogenicity

2. Generate polyclonal antibodies against BclA; evaluate the capacity of these sera to block spore-related activity *in vitro*

3. Evaluate the BclA as a protective immunogen and assess the capacity of the antibodies to prevent anthrax disease in a mouse model

4. Assess the effect of BclA on spore germination, interaction with extracellular matrix proteins, and hydrophobicity

Chapter Two

Recombinant exosporium protein BclA of *Bacillus anthracis* effective as a booster for mice primed with suboptimal amounts of protective antigen

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Note: all of the figures and tables shown reflect work of Trupti Brahmbhatt with the exception of macrophage assays that were performed by Dr. Tae Kang and Dr. Allan Cross. Dr. Patrick Sanz cloned *bclA*. Mr. Stephen C. Darnell and Mr. Humberto Carvalho provided assistance with animal experiments. Dr. Robert Bull assisted with antibody production. Dr. Alison D. O'Brien contributed to the interpretation of data. Drs. Susan Rasmussen and Alison D. O'Brien contributed to manuscript preparation.

ABSTRACT

Bacillus collagen-like protein of anthracis (BclA) is an immunodominant glycoprotein located on the exosporium of *Bacillus anthracis*. We hypothesized that antibodies to this spore-surface antigen are largely responsible for the augmented immunity to anthrax that has been reported for animals vaccinated with inactivated spores and Protective Antigen (PA) compared to PA alone. To test this theory, we first evaluated the capacity of recombinant, histidine-tagged, non-glycosylated BclA (rBclA) given with adjuvant to protect A/J mice against $10LD_{50}s$ of Sterne strain spores introduced subcutaneously. Although the animals elicited anti-rBclA antibodies and showed a slight but statistically significant prolongation in mean-time- to- death (MTD), none of the mice survived. Similarly, rabbit anti-rBclA IgG administered intraperitoneally to mice before spore inoculation statistically significantly increased the MTD but only afforded protection to one of 10 animals. However, all mice that received suboptimal amounts of rPA and then two weeks later rBclA survived spore challenge. Additionally, anti-rBclA IgG, when compared to anti-PA IgG, promoted a 7-fold greater uptake of opsonized spores by mouse macrophages and markedly decreased intramacrophage spore germination. Since BclA has some sequence similarity to human collagen, we also tested the extent of binding of anti- rBclA antibodies to human collagen types I, III and V and found no discernable cross reactivity. Taken together, these results support the concept of rBclA as a safe and effective boost for a PA-primed individual against anthrax and further suggest that such rBclA- enhanced protection occurs by induction of spore-opsonizing and germination-inhibiting antibodies.

INTRODUCTION

Spores of *Bacillus anthracis*, the causative agent of anthrax, are the infectious form of the organism and can persist in soil in a dormant stage for decades (86). Although herbivores are the primary reservoir of anthrax, humans can contract anthrax, albeit rarely, if inoculated with spores cutaneously, orally, or inhalationaly (35). Though anthrax is typically seen only in individuals involved in certain occupations, the potential for infection of larger numbers of people by the aerosol route is of public health concern because of the misuse of *B. anthracis* spores that occurred in the United States in 2001 (36).

One way to protect vulnerable individuals and populations against anthrax is through a strategy of prophylactic immunization. Currently, the anthrax vaccine adsorbed (AVA) is the only licensed anthrax vaccine for use in the United States. AVA is comprised of a formalin-treated, aluminum salts-adsorbed cell-free culture filtrate from an attenuated strain of *Bacillus anthracis* (19). Though AVA is considered safe and effective, the utility of the vaccine is limited by its availability, reactogenicity, a requirement for administration of multiple doses (19), and the generally adverse publicity the vaccine has received (116). AVA is considered to induce protection against anthrax primarily through elicitation of an immune response to the protective antigen (PA) in the preparation (116, 117). Indeed, the strongest correlate of immunity to anthrax, although not considered a perfect association, is the level of antibodies to PA (96). Since PA serves as the cell-binding component for both edema factor and lethal factor (41) and because the lethality of anthrax is primarily attributed to toxemia [as well as septicemia (86)], elicitation of neutralizing antibodies to PA is considered key to induction of

immunity to anthrax. For these reasons, the next generation anthrax vaccine will be based on PA (116, 117). However, the extent of protection provided by purified PA or recombinant PA (rPA) against lethal anthrax infection in several different animal models has proven to be variable (17, 43, 60, 62, 63, 125, 130). Indeed, findings from 2 groups (79, 125), strongly indicate that PA-based vaccines are less protective than live spore vaccines against virulent strains of *B. anthracis*. Moreover, Brossier and colleagues demonstrated that formaldehyde-inactivated spores (FIS) increased the protective efficacy of PA- based vaccines despite similar anthrax lethal toxin-neutralizing activities of the sera from the animals given PA alone versus PA plus FIS (21). Additionally, attenuated nontoxinogenic and nonencapsulated recombinant *B. anthracis* spore vaccines conferred better protection than vegetative-cell vaccines against lethal anthrax spore challenge (26, 61), observations that support a role for spore-associated antigen(s) in protective immunity. Since whole spore-based vaccines are not acceptable for human use in the United States, one alternative approach might be to incorporate individual spore antigens as additives or adjuncts to a PA-based vaccine.

To identify the best such spore antigen(s), we elected to first focus on immunogens that are located on the outermost surface of the spore. *B. anthracis* spores are covered with a loosely fitting balloon-like structure called the exosporium from which hair- like structures project (111). For our study here, we chose to first examine the major component of those exosporium appendages, the glycoprotein BclA. BclA stands for Bacillus collagen-like protein of anthracis and was so named because of similarities in repeat region numbers and amino acids motifs with collagens. BclA is a particularly attractive target because the antibody response generated against spores is primarily

directed against this molecule, specifically to its protein and not carbohydrate constituents (110). Moreover, Hahn *et al.* recently showed that immunization with a combination of PA- and BclA-encoding plasmids conferred significantly better protection than immunization with PA- or BclA- only encoding plasmids in outbred mice challenged with the fully virulent Ames strain of *B. anthracis* (53).

In this investigation, we discovered that A/J mice immunized with non-glycosylated BclA (rBclA) alone or given rabbit anti-rBclA IgG passively exhibited a statistically significant increase in mean-time-to-death after challenge with an otherwise lethal dose of *B. anthracis* Sterne strain spores. Furthermore, we observed that rBclA administered as a boost after primary immunization with suboptimal amounts of rPA conferred full protection against lethal anthrax spore challenge of A/J mice. Possibly as an explanation for this enhanced protection, we also found that rabbit anti-rBclA IgG enhanced opsonophagocytosis of spores by murine macrophages and inhibited intra-macrophage spore germination. Lastly, and most critical for any possible future use of BclA as an anthrax vaccine adjunct or component, anti-rBclA antibodies did not react with human collagens.

MATERIALS AND METHODS

B. anthracis strain, preparation of spores, and spore surface protein extract.

B. anthracis Sterne strain 34F2 was obtained from the Naval Medical Research Center.

B. anthracis was induced to sporulate on Leighton-Doi Medium (LD) (75). The broth

was inoculated with an overnight culture of *B. anthracis* and incubated for 72 hours at

30°C with slow agitation. The spores were harvested by centrifugation, resuspended in

and then washed three times with sterile cold water. Spores were then purified through

Hypaque-76 gradient (Nycomed, Inc., Princeton, NJ), washed three times in cold sterile

water, and stored at 4°C. Prior to use of spores for challenge of animals, an aliquot of the

purified washed spores was heated at 65°C for 30 minutes, diluted, and plated on

trypticase soy agar (TSA) to obtain viable counts. Since heat treatment kills the

vegetative but not spore form of *B. anthracis*, the colony-forming units (CFU) of viable

bacilli recovered after heat treatment reflect the number of spores within the sample.

Spore surface protein extracts (SSPEs) were derived by incubation of 10^9 spores

in 1 ml of buffer that contained 0.1 M L-dithiothreitol, 0.5% sodium dodecyl sulfate,

0.1M NaCl, pH 10.0 at 37°C for two and a half hours, essentially as outlined by Aronson

and Fitz-James (11). The spores were then harvested by centrifugation at 16,000 X *g* for

10 minutes, and the supernatant that contained the SSPE was removed and stored at 4°C.

Preparation of rBclA. Our procedures for construction of a recombinant *E. coli*

strain that expresses rBclA with an N-terminal 6 histidine tag and for purification of that

protein by nickel-affinity chromatography are also described in detail elsewhere

(Brahmbhatt T.N, J.K. Janes, E.S. Stibitz, S.C. Darnell, P. Sanz, S.B. Rasmussen, and

A.D. O'Brien, Inf. Immun., manuscript accepted). In brief, we amplified the sequence of

bclA from the Sterne strain by the polymerase chain reaction (PCR) based on primers

designed from the sequence of the Ames strain [the National Center for Biotechnology

Information (NCBI) web site at

http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=3026019], purified

the PCR product, inserted the DNA fragment into the expression vector pET15b

(Novagen, San Diego, CA), and then sequenced the gene in the resulting recombinant

plasmid to verify that no changes had occurred during the PCR process. A *bclA*-

containing plasmid was then transformed into *Escherichia coli* BL21(DE3) pLysS

according to the pET system manual (Novagen, San Diego, CA). The BclA protein with a

six-His tag was expressed from that transformant and subjected to His-Trap Nickel

affinity column chromatography with the Fast Phase Liquid Chromatography (FPLC)

ÄKTA system (GE Healthcare, Piscataway, NJ).

Rabbit anti- rBclA and anti-PA IgG. Preparation of rabbit anti-rBclA IgG is

also detailed elsewhere (Brahmbhatt T.N, J.K. Janes, E.S. Stibitz, S.C. Darnell, P. Sanz,

S.B. Rasmussen, and A.D. O'Brien, Inf. Immun., manuscript accepted). Briefly, rabbits

were vaccinated multiple times at monthly intervals with 50 ng of purified rBclA in

Freund's complete adjuvant for the first inoculation, and Freund's incomplete adjuvant

for all subsequent immunizations. IgG fractions of the immune sera were obtained by

passage over protein G columns. These anti- rBclA antibodies recognized rBclA, spore-

surface protein extracts of spores, and whole spores.

Human anti-PA IgG was prepared by two separate chromatography steps. First, IgG was isolated from the serum of an individual who had received the United Kingdom-licensed anthrax vaccine by passage of the serum through a protein A column (Pierce, Rockford, IL) per the manufacturer's instructions (66). Second, the anti-PA component of that IgG fraction was isolated by binding to and elution from a PA affinity column. That affinity column was prepared by coupling 1 mg of *B. anthracis* rPA (kindly provided by Dr. Les Baillie, University of Maryland Biotechnology Institute) to 1 ml of EAH Sepharose 4B [GE Healthcare, Waukesha, WI (formerly Amersham Biosciences)] through a carbodimide-mediated coupling reaction. The purified IgG obtained by elution from the protein A column was cycled three times through the washed PA-EAH Sepharose 4B affinity column to separate non-PA-specific IgG from PA-specific IgG. The affinity column was then washed with PBS, pH 7.4, and PA-specific IgG was eluted from the column with 0.15 M glycine-HCl pH 2.5. The acidic fractions were immediately neutralized with 0.15 M Tris.

Animals, immunization, and challenge. Six- to eight- week-old female A/J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and quarantined for 1 week before use. Pre-immune serum samples were collected from mice by tail nicks. The next day, the mice were immunized intraperitoneally (i.p.) with either phosphate-buffered saline, pH 7.4 (PBS), purified rPA (BEI Sciences, Manassas,VA) or rBclA in PBS mixed 1:1 with TiterMax Gold, a water-in-oil adjuvant (TiterMax USA Inc., Norcross, GA) in a total volume of 100 μl. The mice were bled on day 14 and boosted as above on day 15. The mice were bled again on day 28 and challenged subcutaneously on

day 29 with 10 times the 50% lethal dose (LD_{50}) of Sterne spores (about 10^4 spores). The animals were then monitored twice daily for survival through day 15 after challenge.

Enzyme-linked immunosorbent assays (ELISA) to measure anti-rPA and anti-rBclA antibodies. ELISAs were done to assess anti-rPA and anti-rBclA mouse sera IgG responses. Purified rBclA or rPA (100 ng of each in 100 μl PBS) were used to coat the wells of a "U"-bottom 96-well microtiter plate (Thermo Electron Corp., Milford, MA), and the microtiter plates were incubated at 4°C overnight. The microtiter plates were then washed three times in PBS that contained 0.05% Tween-20 (PBST), and the sites on the wells to which antigen had not bound were blocked by incubation overnight at 4°C with 200 μl per well of PBST that contained 5% dry milk. Microtiter plate wells were washed again three times with PBST, and 100 μl of a single 1:100 dilution of each mouse pre- and post-immunization serum sample was then added to the appropriate well of the plate. Each mouse serum sample was tested in triplicate. The microtiter plates were then incubated for 2h at 37°C after which the wells of the plates were washed three times with PBST. Next, 100 μl aliquots of the secondary antibody, goat anti-mouse IgG conjugated to horse radish peroxidase (HRP; Bio-Rad, Hercules, CA) at a dilution of 1:10,000 in PBS, were added to wells that contained mouse serum or positive monoclonal antibodies (see below); the plates were then incubated at room temperature for 1 hour. The secondary antibody was detected with 3, 3', 5, 5' tetramethyl benzidine peroxidase (TMB; Bio-Rad, Hercules, CA), and the microtiter plates were incubated at room temperature for 15 min to permit color development. One hundred microliters of 1M H_2SO_4 was then added to quench the reaction, and the intensity of the yellow in each well

was measured by OD_{450} in an ELISA microtiter reader (Microtek, Molecular Devices,

Sunnyvale, CA). The average intensity of the ELISA reading from the 1:100 dilution of

each post-immune serum sample prepared in triplicate was determined after subtracting

the average OD_{450} readings of triplicate 1:100 dilutions of pre-immune serum from the

same animal. (Note: none of the pre-immune serum samples reacted more than 3-fold

above background). The positive controls for the anti-rPA and anti-rBclA ELISAs were

a monoclonal anti-PA antibody [14B7, originally prepared by Dr. S. Little, and provided

by the Naval Medical Research Center] and rabbit anti-rBclA antibody (IgG), diluted

1:5,000 and 1:10,000 in PBS, respectively.

Passive immunization. For this pre-exposure prophylaxis experiment, 5-6-week-

old female A/J mice (ten per group) were given a single intraperitoneally administered

dose of either 0.1ml of PBS, 0.5 mg in 0.1 ml of anti-rBclA IgG, or normal rabbit IgG 30

to 40 min prior to subcutaneous (back of neck) challenge with $1x10^4$ (~10 LD_{50}) of $B.$

$anthracis$ Sterne spores. All mice were observed for survival twice daily for 15 days

post-challenge.

Effect of anti-rBclA antibody on uptake and killing of spores. Thioglycolate-

elicited peritoneal macrophages from Crl:CD-1 (ICR) BR mice (Charles River,

Wilmington, ME) mice were collected, cultured, allowed to rest overnight, and infected

with spores at a multiplicity of infection (MOI) of 1 as detailed elsewhere (21); the

number of macrophage-associated $B.$ $anthracis$ was determined as described previously

(21). Reagents used for these studies included: RPMI 1640 (Gibco-BRL, Frederick,

MD), fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), gentamicin, and PBS (both purchased from Biosource International, Rockville, MD). Thioglycolate medium brewer modified was obtained from Becton Dickinson (Cockeysville, MD). For these experiments, spores were mixed in 100 μl aliquots (10^6 spores) with an equal volume of 40 μg/ml in RPMI of anti-rBclA IgG, anti-rPA IgG, or 100 μl RPMI alone and incubated at 4°C for 30 min on a rotator. Opsonized spores were then added to the macrophages. The infected macrophages were then incubated at 37°C in 5% CO_2 for 30 min to allow phagocytosis of the treated spores to occur in RPMI medium without fetal bovine serum (FBS) or antibiotics. As an additional control, spores were mixed as above with 20 μl of complement (baby rabbit complement, Cedarlane, Westbury, NY) and then added to the macrophages. The infected macrophages were washed and incubated for another 30 min in the presence of gentamicin to kill any extracellular vegetative *B. anthracis*. The macrophages were then washed further, lysed with cold distilled water, and viable CFU were determined. Aliquots from each sample were also incubated at 65°C for 30 min prior to plate count to ascertain the number of ungerminated intracellular spores. Additional experiments, in which intracellular germination of spores was monitored in macrophages incubated in the presence of gentamicin for an additional 4 or 24 hours, were also done.

Cross reactivity study of rBclA with human collagens. An ELISA was designed to assess any cross reactivity between BclA and human collagen type I, III and V. For this purpose, purified rBclA, spore surface protein extracts, whole spores, human collagen type I, type III or type V (100 ng in 100 μl PBS; purchased from Temecula, CA)

were used to coat the wells of a "U"-bottom 96-well microtiter plate (Thermo Electron

Corp., Milford, MA). These microtiter plates were incubated at 4°C overnight. The next

day, the plates were washed three times in PBST, and then the wells were blocked

overnight at 4°C with 200 µl per well of PBST that contained 5% dry milk. Primary

antibodies for this ELISA included an anti-BclA monoclonal antibody (EG4, Critical

reagent program, Department of Defense, USA), rabbit anti-rBclA IgG (as described

above), an anti-spore rabbit polyclonal antibody (designated 733 and kindly provided by

Dr. Susan Welkos, USAMRIID) that primarily recognizes BclA, and rabbit anti-human

collagen type I, III and V sera (Chemicon, Temecula, CA). Dilutions of these antibodies

(1:10,000 for anti-human collagen types I, V, anti-rBclA IgG, and 733, 1:5,000 for anti-

human collagen type III and EG4) were added to the appropriate wells, and the microtiter

plates were incubated for 2h at 37°C. Antibody dilutions were selected based on the

manufacturer's recommendations (the anti-collagen antibodies) or because they were

several dilutions below the titer against the homologous immunogen (example: anti-

rBclA IgG ELISA titer against rBclA $\geq$1:200,000). The wells of the plates were then

washed three times with PBST. Next, 100 µl of the appropriate secondary antibody, goat

anti-mouse or goat anti-rabbit IgG conjugated to HRP, was added at a dilution of

1:10,000 in PBS, and the plates were incubated at room temperature for 1 hour. Substrate

addition and color development were as detailed above in the description of the anti-

rBclA and anti-rPA ELISA.

RESULTS

Recombinant BclA is immunogenic but not protective, when given alone, against spore challenge. We initiated our search for a recombinant spore protein that might serve as an additive to or a booster for a PA-based vaccine by focusing on rBclA. Our reasons for this selection were two-fold. First, BclA is the most immunogenic protein on the spore surface (109). Second, BclA is in the outermost substance on the exosporium and, therefore, most readily targeted by the host immune system.

When rBclA was injected intraperitoneally with Titermax Gold into A/J mice, all animals developed a strong antibody response as measured by ELISA (average OD_{450} reading for 6 mice by ELISA =1.6; range: 1.0 – 2.1). However, when the same group of animals was challenged with 10 LD_{50} of Sterne strain spores, none of the animals survived, although the mean- time- to- death (MTD) was slightly but statistically significantly prolonged compared to unimmunized controls (Fig. 4). Thus, rBclA alone, though immunogenic, can not function as a vaccine to protect against anthrax in this animal model.

Figure 4

Time to death of A/J mice after immunization with rBclA (alone) followed 4 weeks later by subcutaneous challenge with 10 LD_{50} of *B. anthracis* Sterne strain 34F2 spores (~10^4 spores). The MTD for the vaccinated group was 4.8 days; the MTD for the group given PBS (mixed 1:1 with Titermax) was 4.1 days. This difference in MTD was statistically significant ($P = 0.0045$) by the Kaplan-Meier test. The average anti-rBclA ELISA OD_{450} response for a 1:100 serum dilution of the r-BclA immunized mouse group was 1.6 (data not shown).
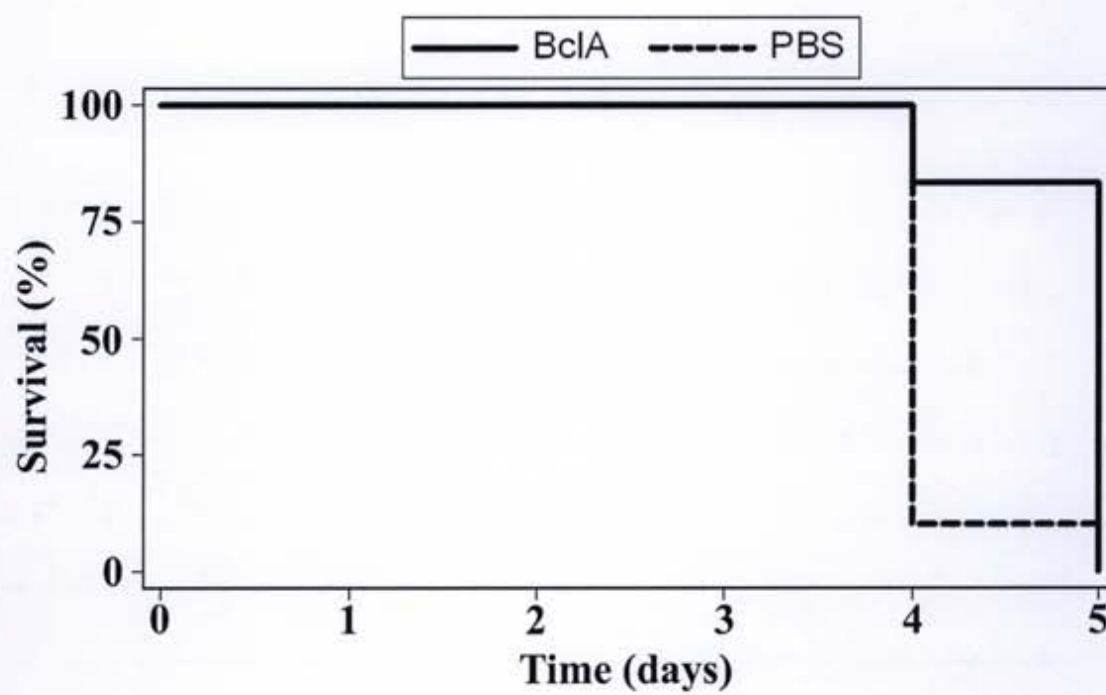
Pre-exposure passive therapy with high-titer rabbit anti-rBclA IgG prolongs mean time-to-death of spore-challenged mice. We then asked whether anti-rBclA, could protect or increase the MTD when given immediately prior to spore challenge. Three groups of 10 mice each were intraperitoneally injected with PBS, 0.5 mg of normal IgG derived from rabbit serum, or anti-rBclA IgG. After a 30-40 minute interval, these mice were challenged subcutaneously with ten times the lethal dose of *B. anthracis* Sterne strain spores. (1×10^4). All of the control mice [one group given PBS and one group given normal rabbit (NRS) IgG] died by day 6 (MTD = 4.3 days for both control groups), but the mice injected with anti-rBclA IgG survived statistically significantly longer [MTD = 6.8 days, $P=0.0002$, as determined by the Kaplan-Meier test)] than did either of the control groups. In fact, 1 of 10 mice administered rabbit anti-rBclA IgG was alive at the time (15 days) that the study was terminated (Fig. 5). Thus, our findings from both active immunization with rBclA and passive administration of anti-rBclA IgG indicate that anti-rBclA antibodies extend survival of mice challenged with anthrax spores.

Figure 5

Impact of administration of rabbit anti-rBclA IgG given one hour before subcutaneous challenge of A/J mice with 10 LD_{50} of *B. anthracis* Sterne strain 34F2 spores (~10^4 spores) on animal survival. The MTD of mice given PBS or passively administrated normal rabbit IgG before spore challenge was 4.3 days for each group compared to 6.8 days for mice that received rabbit anti- rBclA IgG. This difference in MTD was statistically significant as assessed by the Kaplan-Meier test ($P= 0.0002$).
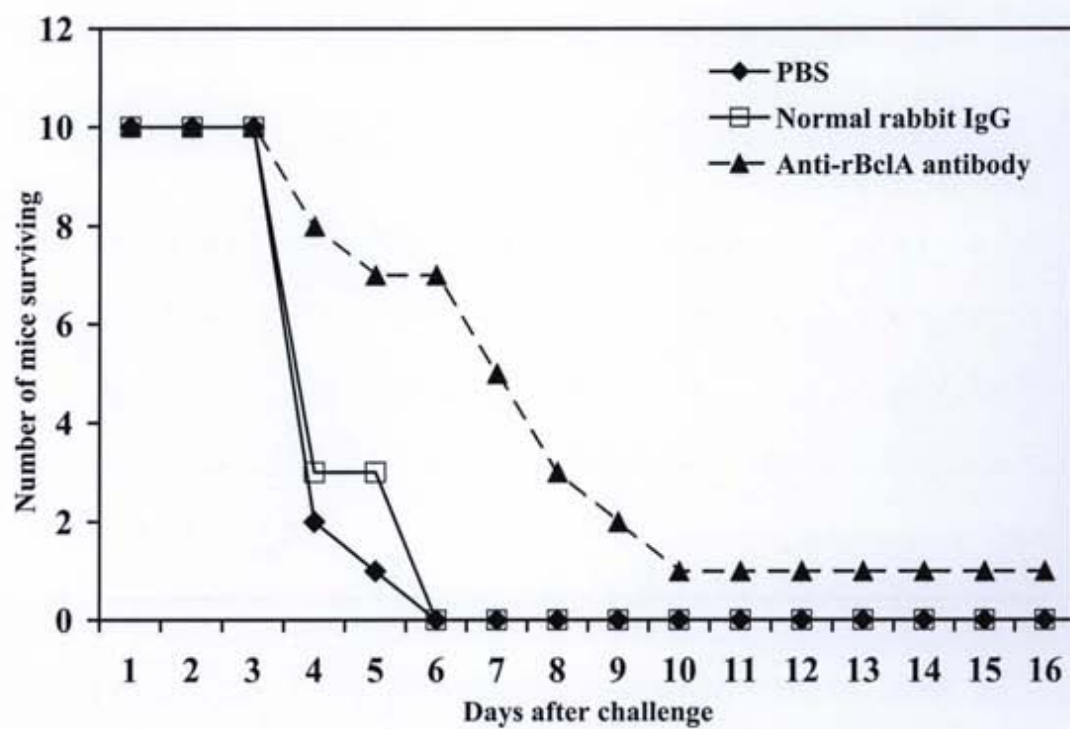
Priming with suboptimal levels of PA followed by rBclA fully protects mice from spore challenge. Next we sought to assess whether mice immunized with a suboptimal concentration of rPA could be protected from anthrax spore challenge by incorporation of rBclA into the immunization schedule. For that purpose, we first immunized groups of mice with different concentrations of rPA and challenged with spores. We determined that a single 50 ng dose of rPA protected approximately 50% of the mice (data not shown) on spore challenge. We then immunized mice with 50 ng of rPA (the suboptimal dose) and 10 ug rBclA. When given with rBclA, rPA-immunized mice did not elicit the same ELISA OD_{450} intensity of anti-rPA antibodies as they did when rPA was given alone (the average anti-rPA OD_{450} reading when rPA was given alone = 1.5; average OD_{450} reading when rPA was given with rBclA = 1.1). Also, and perhaps because of the less intense rPA antibody response, the mice that were immunized with both antigens simultaneously were not protected from anthrax spore challenge (0 / 10 survivors). Next we immunized mice with rPA and boosted at 2 weeks with rBclA. When this immunization schedule was followed, the mice developed an adequate antibody response to rPA (Fig. 6A) as well as antibodies to rBclA (Fig. 6B), and, most importantly, the animals were fully protected (Fig.6C). This rBclA-mediated boost in protection compared to mice given suboptimal amounts of rPA alone was statistically significant, $P = 0.001$. Therefore, we conclude that immunization with suboptimal amounts of the vegetative antigen PA followed by a boost with the exosporium antigen rBclA is a protective anthrax vaccine regime in this mouse model.

Figure 6

Protection of A/J mice primed with suboptimal amounts of PA (mixed 1:1 with Titermax) and boosted with rBclA (mixed 1:1 with Titermax) from subcutaneous challenge with 10 LD_{50} of *B. anthracis* Sterne strain 34F2 spores ($\sim10^4$ spores). A/J mice were injected with 50 ng of rPA or PBS as a control (each mixed 1:1 with Titermax) followed two weeks later with PBS or 10 ug of rBclA (each mixed 1:1 with Titermax). Spores were administered 2 weeks after the rBclA or PBS boost. Panel A: Individual mouse antibody ELISA OD_{450} responses against rPA (Panel A) or rBclA (Panel B). Panel C: Survival curve post subcutaneous challenge with 10 LD_{50} of *B. anthracis* Sterne strain 34F2 spores ($\sim10^4$ spores) ($P=0.001$). Each circle represents the average of triplicate OD_{450} values of a 1:100 dilution of post-immune serum from which average pre-immune values were subtracted for that mouse. Horizontal bars represent the average OD_{450} reading of that immunization group.
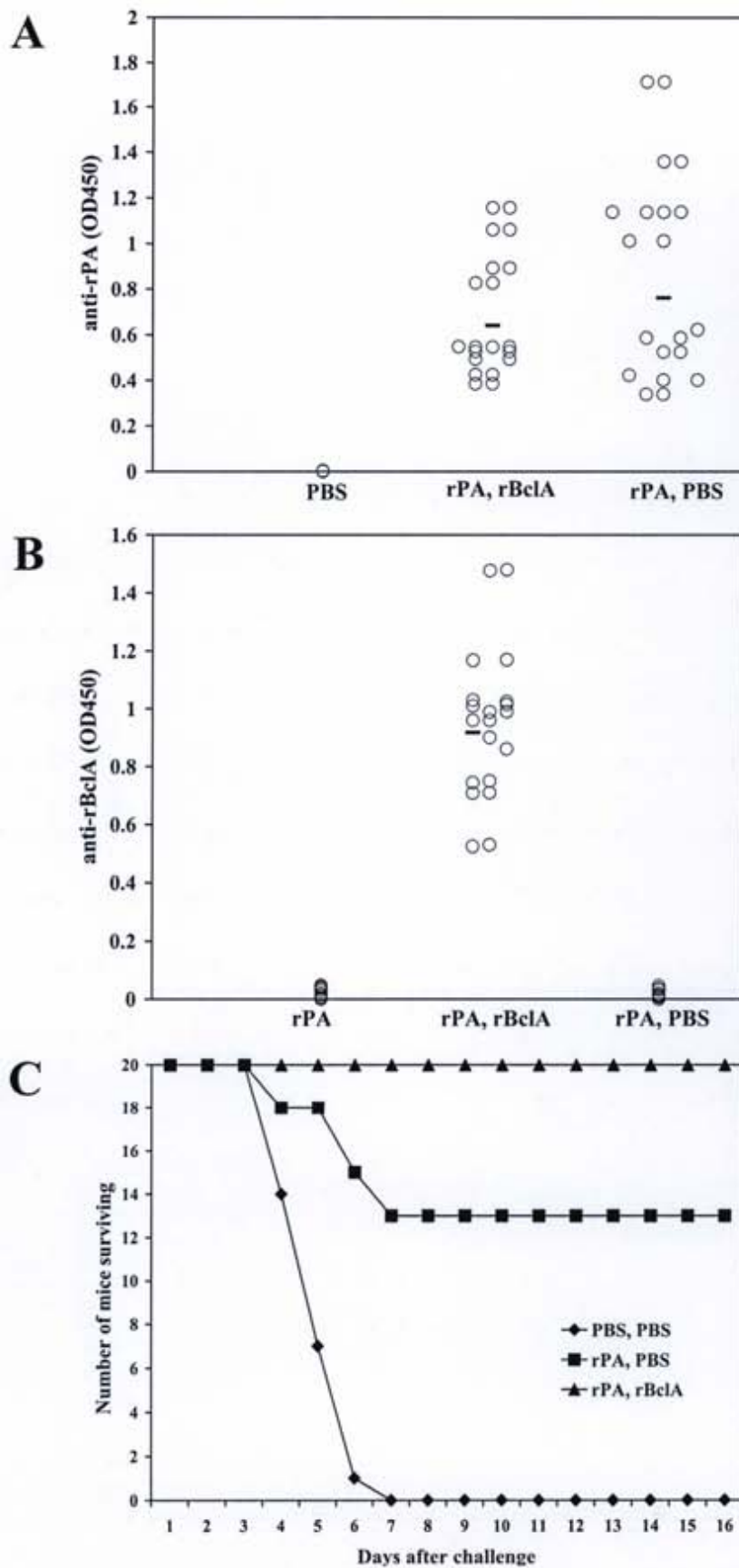
Inhibitory effect of anti-rBclA antibody on germination in macrophages.   *B.*
anthracis spores are believed to germinate within macrophage (50, 54). Since anti-rBclA

antibodies are directed against the outermost component of the exosporium, such

antibodies might contribute to protection against anthrax by functioning as opsonins that

increase uptake and perhaps even killing of spores in macrophages. To test this theory,

we studied the effect of anti-rBclA IgG on the opsonization and germination of spores in

mouse peritoneal macrophages. The macrophages were cultured with *B. anthracis* Sterne

spores (MOI, 1) in the presence of 40 μg of anti-rBclA IgG or anti-PA IgG as a control.

Anti-rBclA IgG increased spore uptake by macrophages 7-fold over anti-PA IgG under

our experimental conditions (Fig. 7A). Moreover, the degree of spore germination within

1 h (i.e., an initial period of 30 min to permit uptake of spores by the macrophages and an

additional 30 min for incubation of the infected macrophages in medium with gentamicin

to kill any extracellular *B. anthracis*) was assessed by heat treatment of the samples

obtained. In the macrophages exposed to spores treated with anti-PA IgG, intracellular

germinated spores represented approximately 40 % of the total interacting spores and was

not different than untreated- or complement- treated spores (Fig. 7B). However, in the

macrophages exposed to spores mixed with anti-rBclA IgG, only 10 % of the

phagocytized spores germinated (Fig. 7B). Furthermore, as shown in Fig. 7C,

intracellular germination of spores opsonized with anti-rBclA IgG remained minimal in

macrophages cultured for 4 and for 24 hours (germination of intracellular

spores/vegetative outgrowth as assessed by heat-sensitive bacterial counts over total

counts , 2% and 3%, respectively, at those time points) as compared to spores treated

with anti-PA IgG (26% & 6%, for 4 and 24 hours of macrophage incubation,

respectively). These results indicate that the anti-rBclA IgG has both an opsonizing and a

sustained germination-inhibiting effect on spores within macrophages maintained *in*

vitro.

Figure 7

Enhanced opsonophagocytosis and killing of *B. anthracis* Sterne 34F2 strain spores by mouse peritoneal macrophages when spores are incubated with rBclA IgG.

Panel A: Uptake by macrophages of spores treated with either anti-PA IgG, anti-rBclA IgG, complement or nothing after one hour of incubation. Panel B: Intracellular germination of spores treated as above after one hour of incubation with macrophages. Panel C: Intramacrophage germination of treated spores over time (1 hour time point shown in panel B included here for estimation of rates). Data in panels A -C were obtained from three independent experiments, each done in duplicate. Data in panels A & B are shown as means ± standard deviation of values.

A

B

C
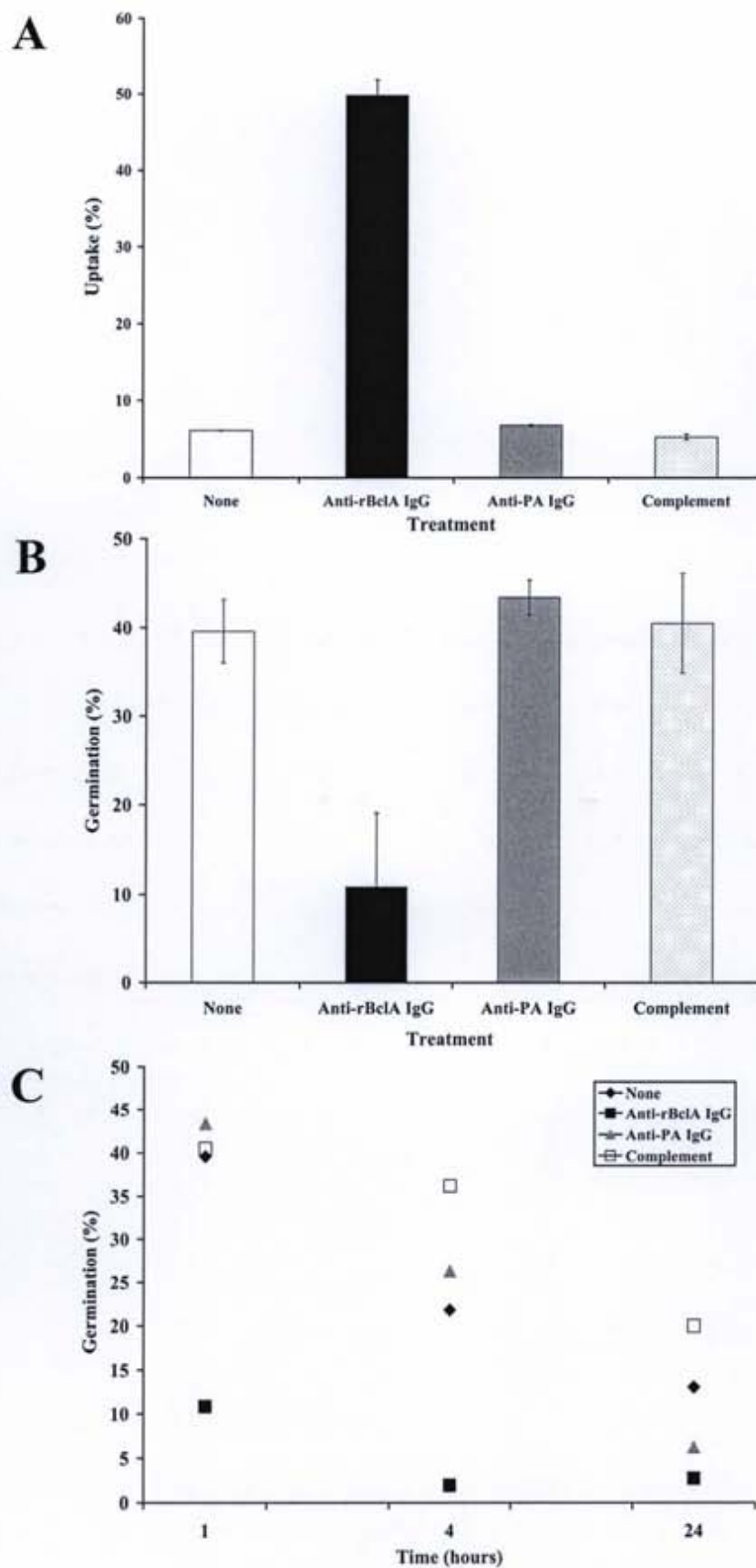
Lack of cross reactivity between BclA and human collagens. Although BclA does not have overall amino acid similarity to human collagen, this exosporium glycoprotein does contain repeated sequences of amino acids that resemble motifs found in human collagen. Indeed, the name Bacillus collagen like protein of anthracis was derived based on these similarities. To address the possibility that BclA and human collagens are cross-reactive, a finding that would rule out any use of the exosporium antigen in a next generation anthrax vaccine, we did a series of ELISA tests. We found that antibodies made by inoculation of spores or rBclA did not cross react with human collagen (Fig. 8A), nor did anti-human collagen (I, III and V) antibodies bind to spores, spores surface protein extract (SSPE) or rBclA (Fig. 8B). We surmise that administration of rBclA to humans should not lead to an auto-immune response to collagens.

Figure 8

Absence of cross-reactivity between anti rBclA antibodies and human collagen.

Panel A: Anti-human collagen (types I, III and V) antibodies did not bind to spores, spores surface protein extract (SSPE) or rBclA.

Panel B: Antibodies made by inoculation of spores or rBclA did not cross react with human collagen types I, III or V. Abbreviations used: RPAb = anti-rBclA rabbit polyclonal antibody; αI = anti-collagen I antibody; αIII = anti-collagen III antibody; and, αV = anti-collagen V antibody; 733 = rabbit anti-spore antibody; EG4 = monoclonal anti-BclA antibody. Samples were tested in triplicate. Bars are shown as means ± standard deviation of values.
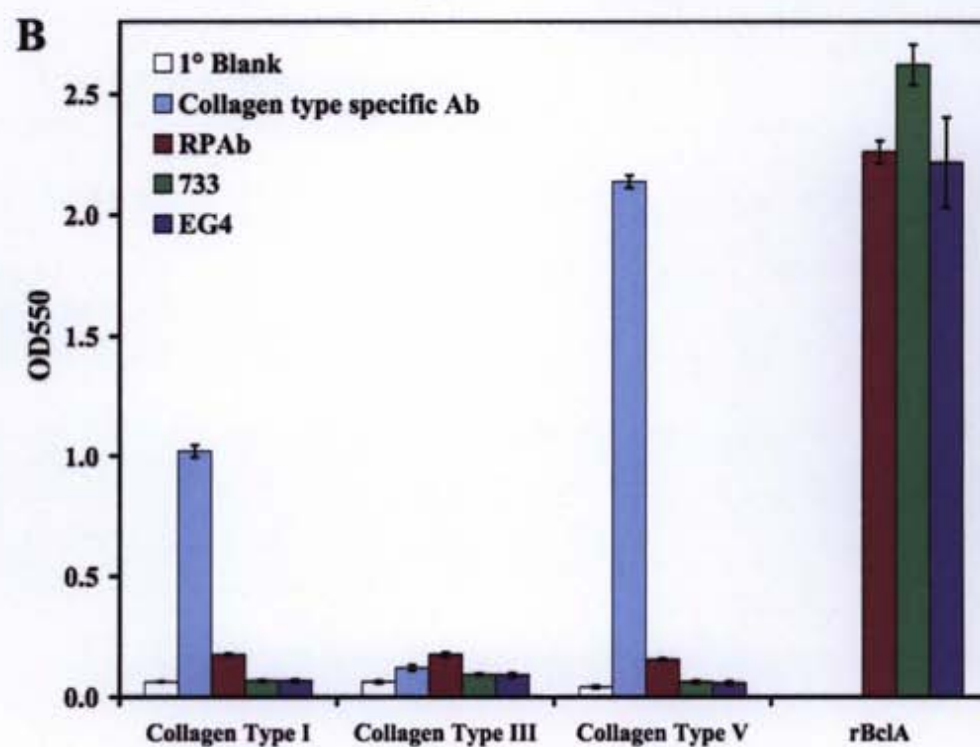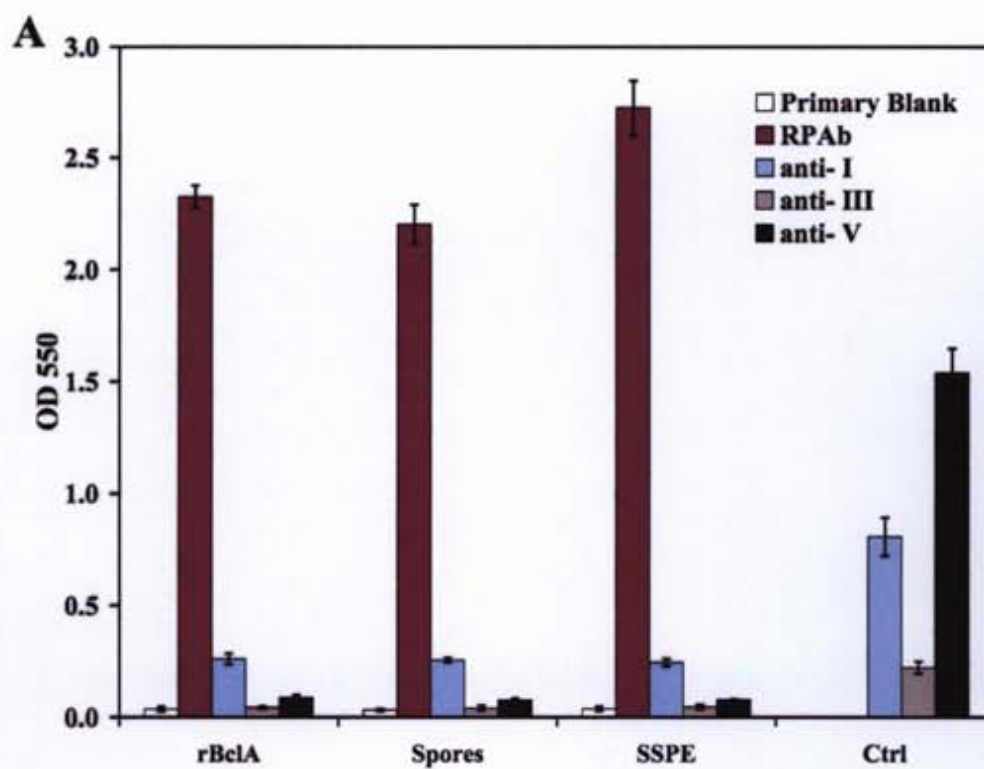
DISCUSSION

The major finding in this study was that a specific spore-surface antigen, rBclA, augmented the protection afforded by rPA against anthrax in a mouse model. The supplementary protection provided by boosting rPA-primed mice with rBclA correlated with the capacity of active immunization with rBclA or passive administration of anti-rBclA rabbit IgG to prolong the MTD of spore-challenged mice. Furthermore, an explanation for the impact of rBclA IgG on immunity was provided by the observation that such antibodies enhance opsonophagocytosis of spores by mouse macrophages while concomitantly decreasing intracellular spore germination. The importance of that latter observation can not be overstated because spore germination within macrophages is considered to be a key step in the pathogenesis of anthrax. In support of the possible use of this spore-surface- based antigen as a boost for a PA-based anti-anthrax vaccine for humans, we detected little or no cross reactivity between rBclA and human collagen types I, III and V.

Our observation that anti-rBclA IgG led to a prolongation in MTD of anthrax-spore-challenged mice is consistent with data from a recent report by Enkhtuya *et al* (42). These investigators demonstrated protection of mice from anthrax by passive immunization of the animals with anti-spore IgG isolated from immune sera of rabbits inoculated multiple times with formalin-fixed spores of a nonencapsulated, non-toxinogenic *B. anthracis* mutant strain. By Western blot, those anti-spore antibodies recognized a number of spore-surface antigens (42) and one of the immunoreactive proteins was consistent in mass with glycosylated BclA (~250 kDa). We speculate that

part if not most of the dose-dependent protection against anthrax spore challenge afforded

the mice passively immunized with anti-spore IgG in the investigation by Enkhtuya and

colleagues (42) likely resulted from the anti-BclA component of that IgG.

The mechanism by which our anti-rBclA IgG prolonged survival of *B. anthracis* spore-

challenged mice was not defined in this study. Nevertheless, based on our *in vitro* data

that showed enhanced uptake and reduced spore germination within murine macrophages

after opsonization with this IgG, we propose the following model. Anti-rBclA antibodies

bind to the hair-like structures on the outside of spores and act as opsonins to enhance

spore uptake by phagocytic cells. Within the macrophages, the tightly bound antibodies

to BclA hinder access of germinants to germinant receptors and decrease the extent of

spore germination. In turn, such anti-rBclA IgG -mediated reduction in intramacrophage

spore germination leads to a delay in vegetative cell formation and, consequently, a later

onset of lethal and edema toxin production and a subsequent extension of the time until

death. In fact, we speculate that if enough antibodies were given before challenge one

might see survival of a substantial fraction of challenged animals, as did Enkhtuya and

co-workers when they administered 0.5 mg. anti-spore IgG per mouse (42). The

observations of Enkhtuya *et al* that anti- spore IgG is opsonic and promotes a reduction in

germination of spores in macrophages are strikingly similar findings to our results in

comparable assays with anti-rBclA IgG. Again, the anti-spore IgG used by Enkhtuya *et*

al and coworkers appeared to recognize BclA, and so had an anti-BclA component.

Lastly, Welkos *et al* previously showed that anti-spore sera reduce the extent of

germination *in vitro* (124). Therefore, our results, taken with the previous observations

of Enkhtuya *et al* and Welkos and colleagues support the idea that anti-spore antibodies,

particularly those directed against the outmost component of the exosporium, can reduce the total load of spores capable of germinating to become toxin-expressing vegetative cells and/or delay the onset of germination.

Our findings with anti-PA IgG as an opsonin of Sterne strain spores reveal, at most, a small anti-germinating effect at 24 hours (but not 1 or 4 hours) of incubation of anti-PA IgG- treated spores in thioglycolate-elicited peritoneal murine macrophages when compared to untreated spores ingested by the same type of macrophages (see Fig.4C). The absence of a significant impact of anti-PA IgG on intra-macrophage spore germination is in contrast to a report by Cote and colleagues (28) who showed that anti-PA antibodies enhanced uptake and germination of Ames spores by RAW 264.7 murine macrophages. The reasons for the discrepancy between their results and ours is not clear but may reflect variation in methods or reagents used by the two groups, e.g., spore strain, method of spore preparation, or type of macrophage used in the assay. Our data are however consistent with previous reports from one of us (66, 97, 123) who used the same type of macrophages and anti-spore germination assay system as was employed in this investigation. Regardless of the reasons for the discordance of our results with the previous report by Cote and co-workers (28) on the anti-germinating capacity of anti-PA antibodies, the findings here indicate that anti- rBclA IgG is a much more effective spore opsonin and anti-germination inducer in macrophages than is anti-PA IgG.

Immunization with rBclA led to a slight but significant prolongation in MTD after spore challenge of A/J mice, but that immunogen alone was not protective. The failure to fully protect animals with such a spore-surface based antigen is not surprising given the requirement in most animal model systems to stimulate anti-toxin immunity through

inoculation with PA, the cell-binding component for edema factor and lethal factor(21).

That we were able to protect mice by first immunizing with suboptimal doses of PA

supports the observation from Brossier *et al* who reported that 100 % of mice immunized

with PA and formaldehyde-inactivated spores were spared from an otherwise lethal

challenge with anthrax spores (21). Additionally, and more directly related to our

findings, Hahn and colleagues reported that immunization with a combination of PA- and

BclA-encoding DNA led to significantly better survival of out bred NMRI mice

challenged with the fully virulent Ames strain spores than did immunization with only

PA- or only BclA-encoding plasmids (53). Nevertheless, the best protection afforded

mice in that study was seen with live Sterne strain spores (avirulent for the NMRI mice

they used in their study) and rPA. That observation not withstanding, Sterne spores,

whether live or irradiated, are not likely to be a component of any future anthrax vaccine

for use in humans in the United States.

Although the methodology used by Hahn and colleagues (53) differed from ours

in several ways, i.e. type of immunogen employed (DNA-based versus protein-based

vaccine), sensitivity of animal model (NMRI mice versus A/J mice), and rigor of

challenge (Ames versus Sterne), their findings and ours led to the same conclusion:

rBclA augments PA-based protection against anthrax spore challenge in a sensitive

animal system. One major difference between our data and theirs is that we only

observed an rBclA-mediated additive protective effect when we gave that immunogen

after inoculation of suboptimal concentrations of rPA. (Note: we used suboptimal doses

of rPA so as to see augmentation of protection; a single higher dose of rPA in A/J mice is

protective). In fact, when we immunized mice with 50 ng of rPA and 10 μg of rBclA

together, the average anti-rPA antibody reactivity, as measured by OD_{450} in an ELISA, was lower compared to the anti-rPA antibody response from mice given rPA alone. We interpreted that result to mean that co-administration of such different concentrations of rPA and rBclA dampened the PA response. Although we did try a number of different regimens [lower doses of rBclA (0.1-5 μg, data not shown)] given simultaneously with 50 ng of rPA, none of the schemes worked as well as our prime with suboptimal PA and boost with rBclA protocol. With this immunization strategy, we not only observed higher antibody responses to rPA but also saw full protection of mice. One potential means of reducing the level of BclA needed (and then perhaps vaccinating concomitantly with PA) is to substitute rBclA with the native glycosylated form of the immunogen expressed from *B. anthracis*. We have initiated studies to express and purify such an immunogen. and also intend to assess the degree of protection afforded by vaccination of more resistant mice than the A/J strain with rBclA or glycosylated rBclA and PA followed by challenge with the fully virulent Ames strain.

For several auto immune diseases, specifically rheumatoid arthritis and systemic lupus erythematosis, common themes are the abnormal appearance of collagen within diseased sites and the presence of anti-collagen antibodies in the patient's serum. Moreover, autoimmune diseases can be induced in experimental animals after exposure to collagen (31). Since BclA shares some sequence patterns with human collagens, we asked whether any anti-human collagen antibodies might be elicited by inoculation of animals with rBclA. We found no evidence that anti-spore- or anti-rBclA antibodies cross reacted with human collagens types I, III, or V. We also observed no reactivity between anti-human collagen antibodies obtained commercially and whole spores, spore

surface protein extracts, or rBclA. In sum, rBclA appears to be a safe spore-associated antigen that could serve as a PA-based vaccine boost, or if conditions for PA and rBclA co-administration can be developed, perhaps even an additional component of a PA vaccine.

Chapter Three

Bacillus anthracis Exosporium Protein BclA Affects Spore Germination, Interaction with Extracellular Matrix Proteins, and Hydrophobicity

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ABSTRACT

The bacillus collagen like protein of anthracis (BclA) is the immunodominant glycoprotein on the exosporium of *Bacillus anthracis* spores. Here, we sought to assess the impact of BclA on spore germination *in vitro* and *in vivo*, surface charge, and interaction with host matrix proteins. For that purpose, we constructed a marker-less *bclA* null mutant in *B. anthracis* Sterne 34F2 strain. The growth and sporulation rates of the Δ*bclA* and parent strains were nearly indistinguishable, but germination of mutant spores occurred more rapidly than wild-type spores *in vitro* and was more complete by 60 minutes. Additionally, the mean time-to-death of A/J mice inoculated subcutaneously or intranasally with mutant spores was lower than for the wild-type spores even though the 50% lethal doses of the two strains were similar. We speculated that these *in vitro* and *in vivo* differences between mutant and wild-type spores might reflect ease of access of germinants to their receptors in the absence of BclA. We also compared the hydrophobic and adhesive properties of Δ*bclA* and wild-type spores. The Δ*bclA* spores were markedly less water-repellent than wild type spores and, probably as a consequence, the extracellular matrix proteins laminin and fibronectin bound significantly better to mutant than to wild type spores. These studies suggest that BclA acts as a shield to not only reduce the ease with which spores germinate but also to change the surface properties of the spore which, in turn, may impede the interaction of the spore with host matrix substances.

INTRODUCTION

Bacillus anthracis is a Gram-positive, spore-forming bacillus that can cause anthrax (86). The spore is the form of the organism found in its natural habitat, the soil, and is also the infectious form for herbivores, the typical vertebrate host for the bacterium, and humans (86). The *B. anthracis* spore is covered by a loose balloon- like membranous structure called the exosporium (47). BclA, (for bacillus collagen like protein of anthracis) was first described by Sylvestre et al (111) who constructed an insertional *bclA* mutant and compared it to its wild-type parent. These investigators, and subsequently others, found that BclA is a glycoprotein and a major component of the hair-like projections that cover the exosporium (98, 109, 111, 115). BclA is also an immunodominant marker on the outside of the spore (109). That BclA does not play a significant role in the virulence of a Sterne-like strain for mice was first reported by Sylvestre *et al* (111). Sterne strains contain pXO1 but not pX02 and are attenuated in humans and many other animals except certain mouse strains (126). In support of the findings of Sylvestre and colleagues, Bozue and coworkers recently constructed a *bclA* mutant of the fully virulent *B. anthracis* Ames strain and showed that the absence of BclA had no impact on the lethality of that strain for guinea pigs or mice(16). Whether BclA, the substance on the spore with which the host cells probably first interact, plays a more subtle role in *B. anthracis* pathogenicity remains to be determined as does the function for this highly expressed glycoprotein in the spore stage of the *B. anthracis* life cycle. Here we report that BclA positive spores, when compared to spores generated from an isogenic marker-less *bclA* mutant of Sterne, display a reduced extent of spore germination by 60 minutes of incubation *in vitro* and a dramatic decrease in the extent of

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association with human extracellular matrix proteins, laminin and fibronectin. We also

show that BclA contributes to spore-surface hydrophobicity.

MATERIALS AND METHODS

Construction of a ΔbclA *B. anthracis* strain. A deletion construct of the *bclA*

gene suitable for allelic exchange was created by cloning two *Not*I-*Xma*I DNA fragments

containing *bclA* flanking regions into the *Not*I site of pBKJ236 (64). These fragments

were created by PCR with the primers: cgcGCGGCCGCagttaggtgcggacagaccttcgaa and

tttCCCGGGtttttttacttagcagtaaaactgat to one side, and

aaaCCCGGGaaattcacctccataaagcgttca and gcgGCGGCCGCacgctttacaagtcgatttcttttg to

the other. Ligation of the two fragments created a precise deletion of the *bclA* open

reading frame, from start to stop codon, inclusive, replacing it with an *Xma*I site. The

resulting construct was used to perform allelic exchange in *B. anthracis* Sterne strain

34F2 (obtained from Dr. Bull, NMRC) to create a *bclA* null mutant by a procedure

described previously (64). In brief, integrants of the *bclA* - pBKJ236 plasmid construct

were isolated by a shift to the replication-nonpermissive temperature after conjugative

transfer and growth at the permissive temperature, while maintaining selection for

erythromycin resistance. A second plasmid, pBKJ223, was then introduced by

electroporation and selection for tetracycline resistance. This plasmid mediates cleavage

within the vector sequences thus stimulating recombination and the loss of the integrated

plasmid resulting in gene replacement in a portion of the erythromycin sensitive

candidates. This strain was named 34F2-ΔbclA. As shown in Fig.1, panel A, absence of

the *bclA* locus in 34F2-ΔbclA was demonstrated by the polymerase chain reaction (PCR)

using the primers 5'-AATCACCGTAATCTTTTGGGTATTG-3' (upstream) and 5'-

CATGGACCTTTCCAAACCATTGAAA-3' (downstream). These primers were

designed to bind to sequences flanking the region included in the deletion construct described above.

Cloning, expression and purification of rBclA. Sequence data for the *B. anthracis* (Ames strain) genome were obtained from The National Center for Biotechnology Information (NCBI) web site at http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=30260195. Based on this sequence, DNA primers were designed to amplify the entire bclA open reading frame (ORF) of the Sterne strain by the polymerase chain reaction (PCR). The sequences of primers were: 5'CACACACATATGTCAAATAATAATTATTCAAATGG3' and 5'CACACAGGATCCTATTAAGCAACTTTTTCAATAATAATGGATGC3'. Genomic DNA was extracted and purified from a 3 ml overnight culture with the Easy DNA kit (Invitrogen, Carlsbad, CA). A 10 ng sample of genomic DNA was added to a PCR mixture as a source of template DNA. PCR amplification was done with High Fidelity DNA polymerase (Roche, Indianapolis, IN). The PCR conditions were 94°C for 5 min; 94°C for 30 s, 50°C for 30 s, and 68°C for 1.5 min for 45 cycles; and finally 68°C for 10 min. The PCR product was purified by agarose gel electrophoresis with Geneclean (Qbiogene, Irvine, CA), digested with *Nde*I and *Bam*HI restriction enzymes (Roche, Indianapolis, IN) and inserted into the corresponding cloning sites of the expression vector pET15b (Novagen, San Diego, CA). The inserted DNA regions in the resulting recombinant plasmids were sequenced to confirm that no changes had occurred during the PCR process. A *bclA*-containing plasmid was transformed into *Escherichia coli* BL21 (DE3) pLysS to express the cloned gene according to the pET system manual (Novagen, San Diego, CA). The expressed BclA protein, with a six-His tag, was purified

by His-Trap Nickel affinity column chromatography using the Fast Protein Liquid Chromatography (FPLC) system of GE Healthcare (Piscataway, NJ).

Immune sera and antibody preparation. Immune sera from rabbits were obtained from animals vaccinated multiple times at monthly intervals with 50 ng of purified rBclA in Freund's complete adjuvant for the first inoculation, and Freund's incomplete for all subsequent immunizations. IgG was purified using a protein G column. Protein concentrations were determined by a microtiter Bicinchoninic Acid (BCA) assay (Pierce, Rockford, IL).

Spore preparation. *B. anthracis* wild-type and 34F2-$\Delta bclA$ vegetative cells were induced to sporulate by growth at 30°C for 3-4 days on Modified G Medium (MGM) (67) that had been rendered solid by addition of 0.5% Difco agar. Spores were scraped from the agar plates and then purified through a Hypaque-76 gradient (Nycomed, Inc.; Princeton, NJ).

Sporulation assays. Two approaches were used to compare the degree of sporulation over time of *B. anthracis* wild-type and 34F2-$\Delta bclA$ vegetative cells over time. For each of these methods, vegetative cells were cultured in Luria Bertani broth for 5 days at 37°C with 100 RPM agitation. For the first protocol, the OD_{600} of the culture was monitored at 24, 48, 72 and 96 hours, a measurement that reflects the density of the particles in suspension. Since spores are less dense than vegetative cells, the OD_{600} should decrease as the bacilli in the culture become spores (104). For the second technique, samples were obtained at the time points listed above and assessed by phase microscopy for sporulation.

Spore surface protein extraction and Western blot analysis. Spore surface protein extracts (SSPEs) were prepared from the wild-type Sterne 34F2 and 34F2-$\Delta bclA$ spores as outlined by Aronson and Fitz-James (11). Briefly, 10^9 spores were incubated with 1 ml buffer that contained 0.1 M DTT, 0.5% SDS, 0.1M NaCl, pH 10.0 at 37°C for two and a half hours. The spores were then centrifuged at 16,000 X g for 10 minutes. The supernatant was removed and stored at 4°C. SSPEs were diluted in sample loading buffer and loaded on 8% Tris-Glycine SDS-PAGE gels, electrophoresed, and transferred onto nitrocellulose. The resulting blots were probed with rabbit anti-BclA (V683) antiserum at a 1:5,000 dilution. Blots were developed using the ECL Western blot analysis system (GE Healthcare) with the secondary antibody at 1:10,000 (Bio-Rad). The primary antiserum (anti-rBclA V683) used for this Western analysis was generated by immunizing rabbits with rBclA as outlined above.

Lethal dose assays. To study the effect of our $bclA$ null mutation on virulence of spores, we used the sensitive A/J mouse model (126). Six -eight week old A/J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and quarantined for 1 week before use. The mice were then inoculated via intranasal and subcutaneous routes with heat-treated spores of either 34F2 or 34F2-$\Delta bclA$. Mice were monitored for 14 days for survival. The 50% lethal dose (LD_{50}) of each B. anthracis strain in the A/J mouse was calculated by the method of Reed and Muench (99).

Spore germination procedure. Spores were heated at 65°C for 30 minutes, inoculated into brain heart infusion (BHI) broth and incubated at 37°C degrees with slow agitation. Samples were taken at 0, 15, 30, 45, and 60 minutes, heat-treated at 65°C for

30 minutes, and dilutions were plated on Trypticase Soy Agar (TSA) plates. After

incubation of the plates at 37°C for approximately 8 hours, the colonies were counted.

Assay to assess host matrix protein binding to spores. To evaluate the relative

degree of interaction of wild-type versus *bclA* mutant spores with fibronectin and

laminin, we developed a binding assay based on a method described by Kuusela *et al*

(74). These researchers examined the attachment of staphylococci and streptococci to

fibrinogen bound to microtiter wells. We made two major modifications to the format of

their protocol: we used an ELISA rather than a direct radioimmunoassay, and we allowed

spores to coat the wells of a microtiter plate first and then added the matrix proteins

second rather than vice versa. The reason for the latter configuration was that such a

design gave the most consistent results in preliminary studies. Therefore, in our assay,

we coated wells with spores at 37°C for 1 hour, washed the wells with phosphate

buffered saline with 0.1 % Tween-20 (PBS-T), and then blocked the wells with 5% non-

fat dry milk in PBS-T for 1 hour. After washing the wells of the plates three times with

PBS-T, either fibronectin or laminin were added at varying concentrations. The plates

were then incubated for 1 hour at 37°C and the wells washed again as above. Next rabbit

anti-fibronectin or anti-laminin antibodies (Sigma-Aldrich, St. Louis, MO) at a dilution of

1:10,000 in PBS-T were added to the wells. The plates were then incubated for 1 hour,

and the wells washed as above. Horseradish peroxidase- conjugated goat anti-rabbit sera

(Bio-Rad) at a 1:10,000 dilution was then added to the wells. After a 45 minute

incubation, the plates were then washed and the 3, 3', 5, 5' tetramethyl benzidine

substrate (TMB; Bio-Rad) added to the wells. After 15 minutes, the reaction was stopped

with 1N H_2SO_4, and the plates were read at 450 nm on ELX-800 (Bio-Tek Instruments

Inc.) plate reader. The assay was done in triplicate, and all control wells reacted as expected. Specifically, wells that contained spores only did not react with anti-fibronectin or anti-laminin antibodies, but fibronectin and laminin were recognized by the specific primary antibody and not by the secondary antibody. Additionally, neither fibronectin nor laminin bound to any significant degree to microtiter wells treated with buffer alone [OD_{450} = 0.05 for binding of laminin and fibronectin (at the highest concentrations used in the assay) to buffer-treated wells]. The latter results indicate that non-specific adherence of the matrix proteins to the buffer-treated plastic wells did not occur as the assay was configured.

Measurement of hydrophobicity of spores. The relative hydrophobicities of spores can be measured by a number of established techniques that include adherence to hydrocarbons (71, 101, 128), hydrophobic interaction chromatography (HIC) (37, 59), salt aggregation (113), and contact angle measurements (74). To investigate the role of BclA in surface hydrophobicity, we compared spores with and without BclA (wild-type versus *bclA* mutant spores) in the bacterial adherence to hydrocarbon (BATH) assay as described by Rosenberg *et al* (101). For the BATH technique, spore suspensions in sterile water were prepared at turbidities of 0.4 - 0.6 at 440 nm absorbance and then mixed with a nonaqueous solvent. Adherence to the hydrocarbon was measured by loss of turbidity in the aqueous phase. A ratio of 0.133 ml hydrocarbon per ml of spore solution is reported to be sufficient for maximal adherence (37). Here we used 0.01, 0.05, 0.1 and 0.2 ml hexadecane/ml spores or 34 - 570 μM hexadecane. The suspensions were vortexed for 30 seconds, and the phases were allowed to separate by standing for 15 minutes. The aqueous phase was carefully removed from the mixture, and turbidity of the aqueous

phase was then measured. The percent hydrophobicity was calculated by the formula:

$(100 - [(A_{after} / A_{before}) \times 100])$.

A second approach, Hydrophobic Interaction Chromatography (HIC) with Sepharose CL-4B as described by Ismaeel *et al* (59), was also used to examine differences between the hydrophobicities of wildtype and $\Delta bclA$ spores. In brief, columns of CL-4B Sepharose were prepared to a height of 1.7 cm in wide-bore Pasteur pipettes plugged with glass wool, and the columns were washed extensively with 4 M sodium chloride buffered to pH 7.0 with 0.01 M sodium phosphate. The spores were suspended in the same buffer, and 5 mls of this mixture was loaded onto the column. The eluate was collected, and the absorbance was measured and compared to that of the applied spore suspension.

Assessment of effect of heat on spore hydrophobicity. To examine the impact of heat on relative hydrophobicity of the wild type and mutant spores, we first had to test the effect of heat treatment alone on spore survival over time. For that purpose, spores of 34F2 or 34F2-$\Delta bclA$ in sterile water were sampled for colony counts (as below) and then subjected to 75°C heat. Aliquots were drawn every hour, diluted and plated in triplicate on TSA plates. After incubation of the plates at 37°C for approximately 8 hours, the colonies were counted. We then used the method described by Wiencek *et al* (128) to measure the impact of heat on hydrophobicity. In brief, spore suspensions were heated in glass tubes at 37, 75, 85 or 100° C for 10 minutes. The heated spores were then rapidly cooled on ice and refrigerated overnight. The hydrophobicities of heated spores were assessed by the BATH assay as described above (with 0.1 ml hexadecane).

RESULTS AND DISCUSSION

We first constructed a *bclA* deletion mutant of *B.* anthracis Sterne strain 34F2. This deletion cleanly excised the entire *bclA* open reading frame, replacing it with an *Xma*I site (CCCGGG). Thus, this mutation was not expected to exert polarity on downstream genes. It should be noted as well, that, although an examination of the annotated genomic sequence of *B. anthracis* Ames indicates the presence of additional genes downstream of, and in the same orientation as, *bclA*, the presence of a predicted rho-independent terminator just downstream of *bclA* also supports the view that this deletion does not affect the expression of downstream genes.

Next, we tested spore surface protein extracts of the wild-type and 34F2-$\Delta bclA$ spores for the presence or absence of BclA by Western blot analysis (Fig. 9, panel B). We observed a broad high molecular weight band in the SSPE from 34F2 spores. That band (~ 180kDa) was smaller but similar in size to that reported for glycosylated wild-type BclA (~250kDa) by Sylvestre *et al* (111). Our *E. coli*-derived rBclA control migrated at ~70kDa, a size consistent with that noted previously by Steichen *et al* (109) for rBclA . As anticipated from the manner in which the 34F2-$\Delta bclA$ mutant was derived, we detected no BclA band in the SSPE of 34F2-$\Delta bclA$.

We then compared the growth and sporulation capacities of the wild-type 34F2 with 34F2-$\Delta bclA$. We found that the strains grew with comparable kinetics and to indistinguishable levels (Fig. 9, panel C). To evaluate the sporulation capacity of the wild-type and mutant strains, spore formation over time in broth culture was assessed by both optical density measurement at 600nm (Fig 9, panel D) and phase contrast

microscopy of samples taken at 24, 48, 72 & 96 hour time points (data not shown).

Results by both sporulation assessment methods were similar for the wild-type and

mutant strains.

Figure 9

Characterization of 34F2-ΔbclA. Comparison of mutant and wild-type strains by PCR using primers that flank the bclA gene as described in Materials and Methods. The predicted sizes of the diagnostic fragments are 2380 bp and 1237 bp, respectively, for the wild-type or deleted bclA locus (Panel A). Western blot of purified rBclA expressed from E. coli, SSPE from wildtype 34F2 and 34F2-ΔbclA probed with rabbit anti-rBclA (V683) antiserum (Panel B). Note that the difference in mobility between rBclA (Panel B, Lane 1) and BclA contained in Sterne strain spore extracts (Panel B, Lane 2) is likely due to glycosylation of BclA in wildtype spores. Sporulation is shown as a measure of optical density (OD_{600nm}). The growth rate and sporulation of the mutant and wild-type strains are depicted in Panels C and D, respectively. T_0 is defined as the time of inoculation of the broth culture with bacteria. The change in size as the vegetative rod (large particle) becomes a spore (small particle) corresponds to a reduction in the optical density as sporulation occurs.

A

Stds.
34F2
34F2-Δ*bclA*

3.0 —
2.0 —
1.5 —
1.0 —
0.5 —

B

rBclA
34F2
34F2-Δ*bclA*

kDa
202 —
115 —
98 —
51 —
37 —

C

D
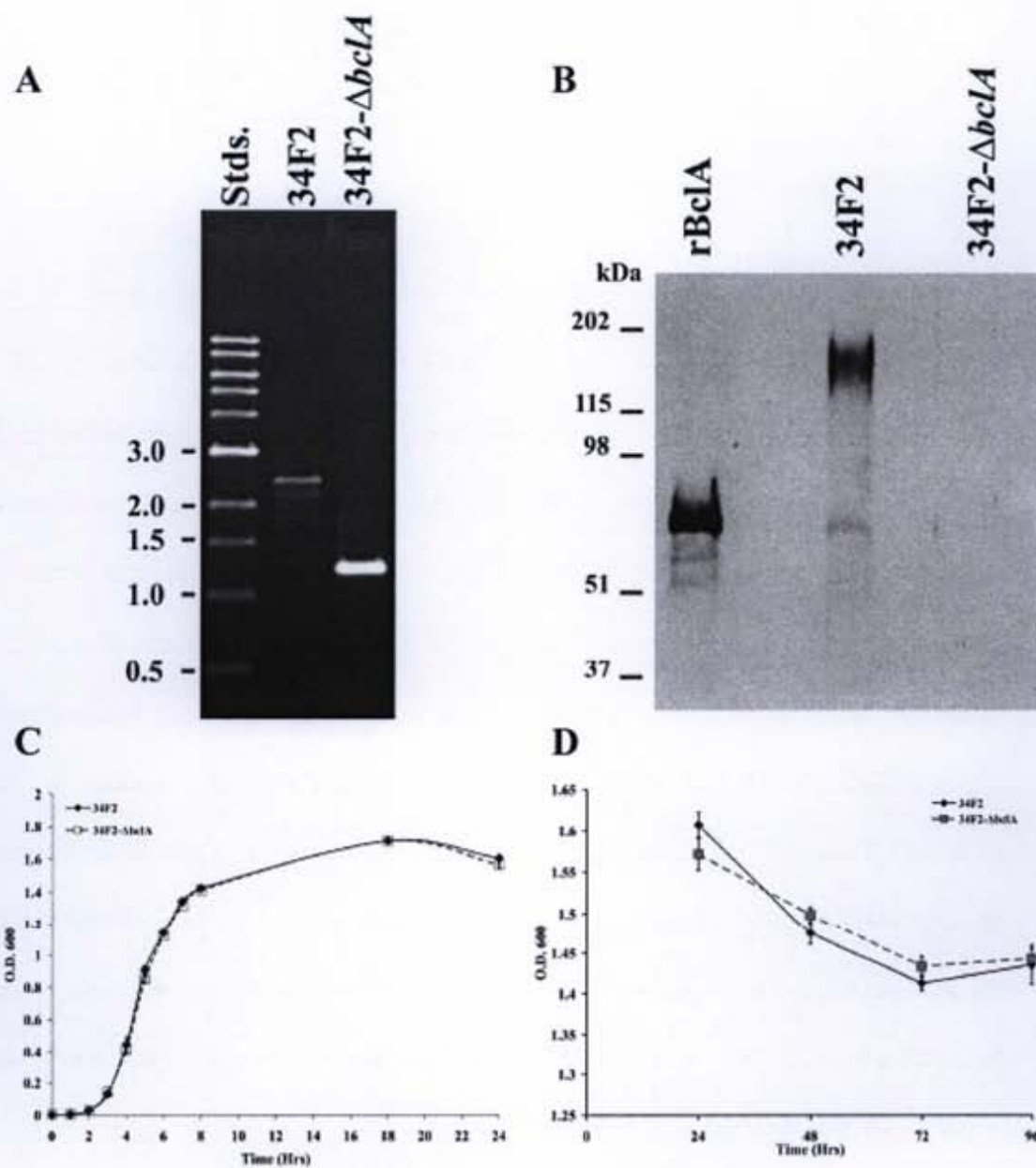
Figure 10

Germination curve of 34F2 and 34F2-$\Delta bclA$ spores. Germination is measured here as loss

of heat resistance. The experiment was repeated three times. ANOVA analysis showed

statistically significant differences (P=0.001 and 0.007 for time points 45 and 60 minutes,

respectively) in the extent of germination between wild type and mutant spores. In the

ANOVA analysis, three experiments were combined and then adjusted for variation.
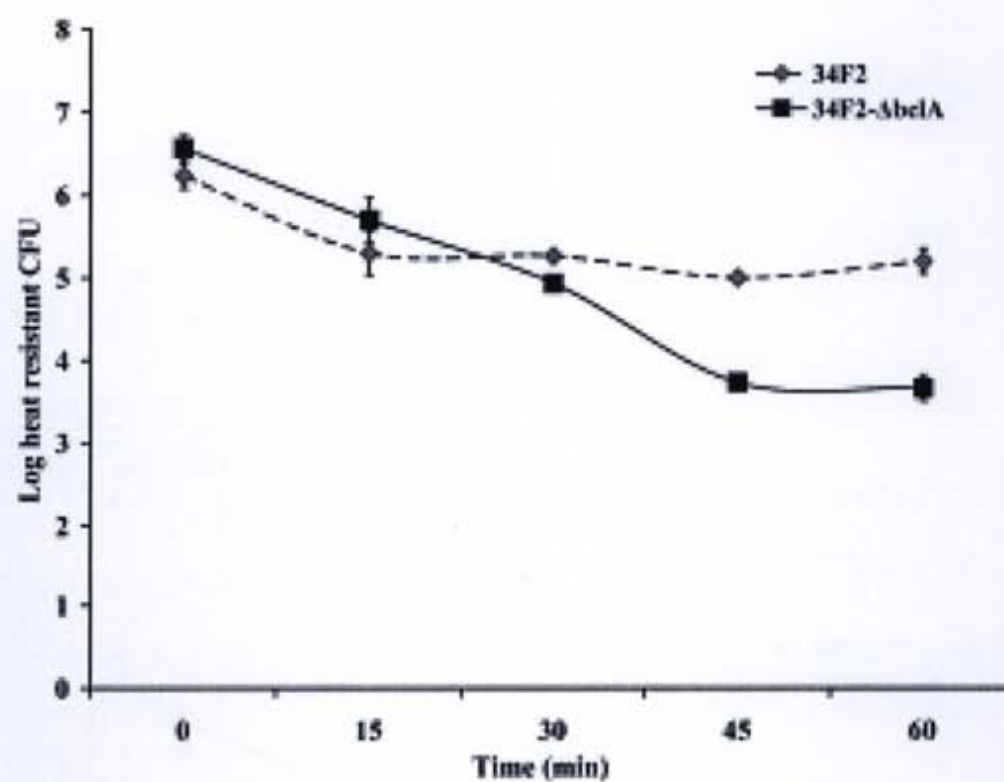
When we compared the relative LD_{50}s of mutant and wild-type spores by intranasal or subcutaneous routes of administration, no statistically significant differences between the 34F2 and 34F2-$\Delta bclA$ were noted (Table 3). This finding is in keeping with the reports of Sylvestre $et\ al$ (111) with a Sterne-like strain and Bozue and colleagues with Ames (12). However, the LD_{50} values we obtained were always lower for the mutant strain (Table 3). Furthermore, the MTD of mice given a 10^6 dose of wildtype 34F2 spores was 4.2 days for the intranasal route and 4.4 days for the subcutaneous route compared to 3 days for mice that received 34F2-$\Delta bclA$ spores by both the intranasal and subcutaneous routes. Thus, the MTD was shorter for the 10^6 dose by both the subcutaneous and intranasal routes for the mutant strain (P=0.006). These two findings taken together may indicate that 34F2-$\Delta bclA$ spores germinate slightly better/faster in $vivo$ (as they appear to do $in\ vitro$) or perhaps associate with host cells more avidly than do wild-type spores. Indeed, Bozue $et\ al$ reported less efficient recovery of $\Delta bclA$ spores of Ames strain from lungs of mice upon bronchio-alveolar lavage at days 2 and 4 in an aerosolized spore challenge (16). The authors speculated that this reduced clearance of the mutant spores from the lungs of these animals might reflect better binding of mutant spores than wild-type to host cells lining the lungs or airways. In fact, very recently, Bozue $et\ al$ demonstrated that $bclA$ mutant spores adhered much better to epithelial cell lines than did wild-type Ames spores. However, these investigators noted that such adhesion differences between their BclA Ames mutant strain and Ames did not extend to macrophages.

Table 3

Comparison of lethal dose 50% (LD_{50}) of spores of *B. anthracis* Sterne strain 34F2 and

34F2-$\Delta bclA$.

Spores source	Route of infection	Experiment #	LD$_{50}$
34F2	Subcutaneous	1	8.4×10^2
		2	6.3×10^2
34F2-$\Delta bclA$	Subcutaneous	1	3.0×10^2
		2	1.3×10^2
34F2	Intranasal	1	6.8×10^4
		2	7.9×10^4
34F2-$\Delta bclA$	Intranasal	1	3.5×10^4
		2	4.0×10^4

To evaluate the possibility that BclA might modulate the surface of the spore in a manner that influences the degree of association of spores with host matrix proteins, we compared how well fibronectin and laminin bound to wild-type and *bclA* mutant spores We found that the interaction of both fibronectin and lamina with 34F2-$\Delta bclA$ spores was more extensive than with 34F2 spores (Fig. 11A and B). These results suggest that when BclA is removed from the surface of the spores other protein(s) may become exposed and adhere to the two extracellular matrix proteins tested. Moreover, our data on the greater stickiness of the ECM components laminin and fibronectin to BclA-negative spores is consistent with the aforementioned report by Bozue *et al* on the enhanced adhesion of *bclA* mutant Ames spores to epithelial cells compared to the parental strain.

Figure 11

Comparison of adherence of matrix proteins to 34F2 and 34F2-Δ*bclA* spores Panel A.

Binding of fibronectin to spores; Panel B. Binding of laminin to spores. Data are shown

as means ± standard deviation of values obtained from 3 experiments done in triplicate.
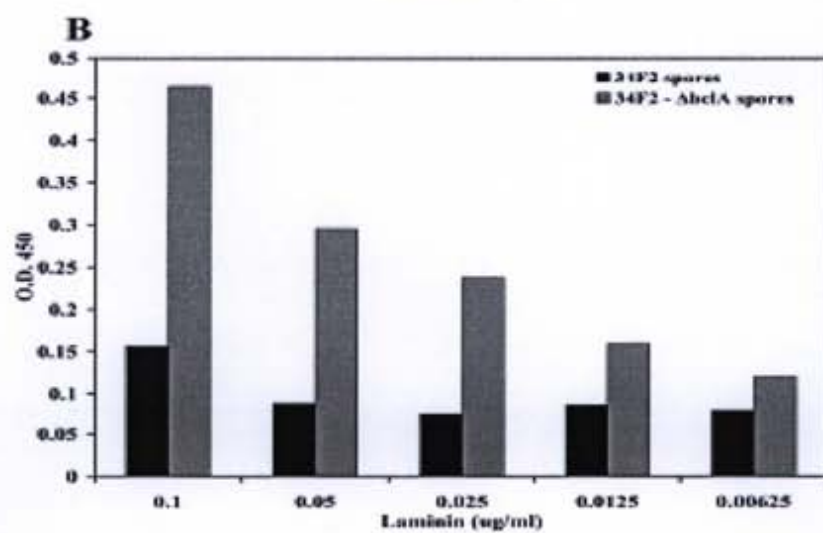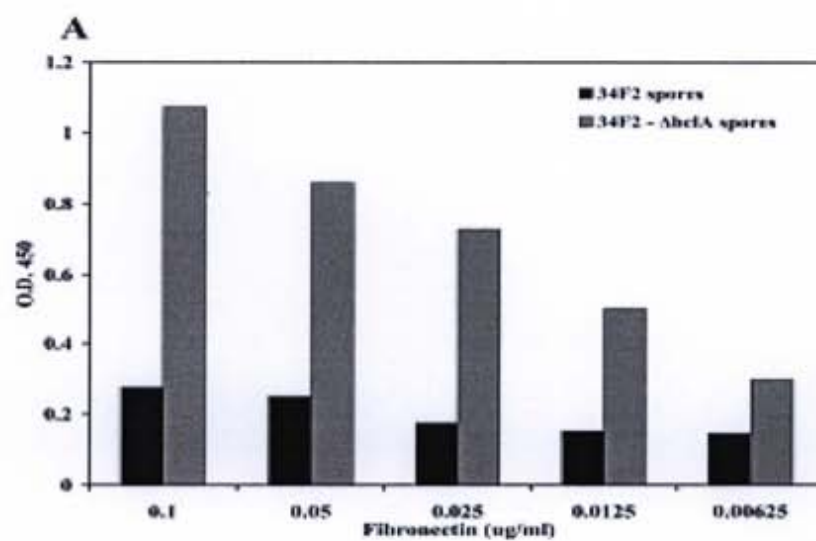
One possible explanation as to why matrix proteins bound less well to BclA positive spores than to BclA-negative spores is that BclA affects the hydrophobic nature of the spore. That bacterial spores are hydrophobic has been demonstrated for several species of *Bacillus* and *Clostridia* (128), and, probably as a consequence, these types of spores adhere strongly to inanimate surfaces like microtiter plates (7). Moreover, *B. cereus* spores bind to Caco-2 cells through surface hydrophobic interactions (10). That the degree of hydrophobicity of spores may be linked to the presence of an exosporium was first suggested by Takubo *et al* (113) who showed that *Bacillus megaterium* QMB1551 spores with a defective or absent exosporium exhibit reduced affinity for hexadecane. Moreover, spores of *B. subtilis*, *B. licheniformis*, and *B. macerans* do not have distinct exosporium layers and are less hydrophobic than are exosporium-containing *B. cereus, B. brevis* and *B. thuringiensis* spores (71). Furthermore, a reduction in partitioning to hexadecane was successfully used by Bailey-Smith *et al* to enrich for exosporium mutants of *B. cereus* (12). Thus, several studies indicate that the surface hydrophobicity of certain spores is attributable to the presence of an exosporium. However, the specific structural component(s) that contributes to the hydrophobic nature of the spore is not known.

To test our theory about the impact of BclA on hydrophobicity of spores, we evaluated the percent hydrophobicity of spores at various molarities of hexadecane. We found that the hydrophobicity of wild type spores was greater than that of $\Delta bclA$ spores at each of the four hexadecane concentrations selected (Fig. 12A). We confirmed the findings of this BATH assay with a second hydrocarbon, toluene at 0.1 ml per ml of spore suspension (853 μM toluene). Again, the wild type spores showed higher

hydrophobicity with toluene than did mutant spores (Fig. 12B). The 34F2 spores were more hydrophobic by HIC than were 34F2-Δ*bclA* spores (data not shown) although the absolute hydrophobicities of the spores as measured by the BATH and HIC techniques, did not agree.

Figure 12

Comparison of hydrophobicity of 34F2 and 34F2-$\Delta bclA$ spores.

Panel A. Hydrophobicity measured with various volumes of hexadecane. Panel B.

Hydrophobicity differences between 34F2 and 34F2-$\Delta bclA$ spores compared with a

second hydrocarbon, toluene. Data represent the average of two experiments.

A

B
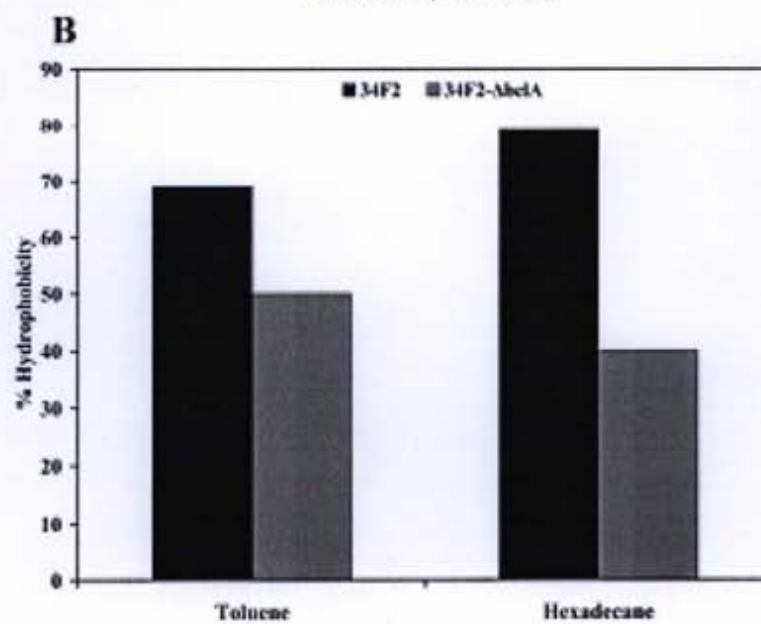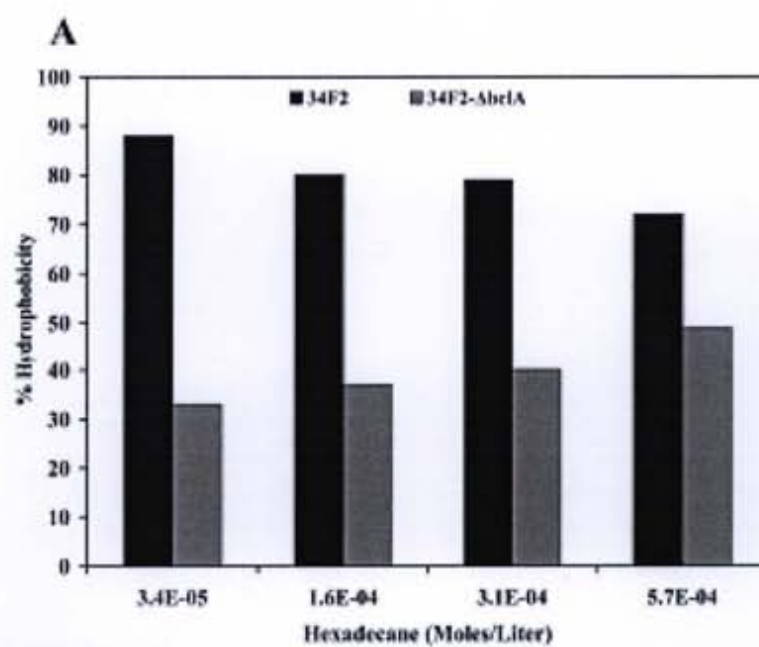
Next we assessed whether the greater hydrophobicity of wild-type versus *bclA* mutant spores would still be evident in the presence of heat. We asked this question because Howell *et al* (55) reported that increased temperatures can alter the structure of macromolecules and expose internal hydrophobic moieties. Moreover, Doyle *et al* suggested that increases in the hydrophobicity of spores because of heat treatment may result from the disruption of outer coat or exosporium proteins (37). To examine the impact of heat on relative hydrophobicity of the BclA positive and negative spores, we first had to test the effect of heat treatment alone on spore survival over time. No differences were noted in relative viability of wild-type and mutant spores after this heat treatment (Fig 13A). We then used the method described by Wiencek *et al* (128) to measure the effect of heat on hydrophobicity. The values obtained were compared with the values determined for the unheated controls, and the results represented as percent increase in hydrophobicity. As shown in Fig. 13B, the mutant spores became strikingly more hydrophobic in the presence of heat than did wild-type spores even though heat did not differentially impact the viability of mutant and wild-type spores (Fig 13A). One interpretation of the findings in Fig. 13B is that spore proteins that are normally obscured by BclA are exposed in its absence and that these now-uncovered proteins are altered by heat treatment in a manner that leads to increased hydrophobicity of mutant spores compared to wild-type.

Figure 13

Evaluation of the impact of heat on hydrophobicity of 34F2 and 34F2-Δ*bclA* spores.

Panel A. Effect of heat treatment on survival. Panel B. Percent increase in hydrophobicity

compared to unheated controls. Both experiments were done in duplicate, and averages

are presented.

A

B
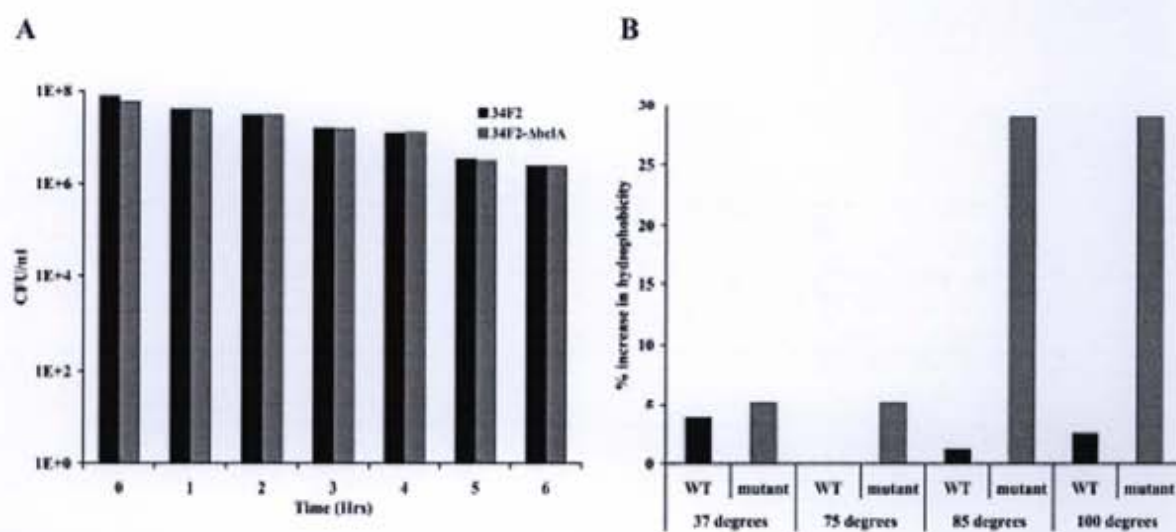
Further insight into how BclA affects the *B. anthracis* spore surface is important for at least two reasons. First, such information may lead to a better understanding of the initial interaction of the spore with its host. Indeed, our data on a shorter MTD of 34F2-$\Delta bclA$ versus 34F2 at a challenge dose of 10^6 spores, taken with a lower, albeit not statistically significantly different, LD_{50} of the BclA mutant versus wild-type strain for A/J mice hint that BclA may slow spore germination *in vivo*. That others have not reported any impact of BclA on virulence of *B. anthracis* spores (16, 111) may reflect the subtlety of a BclA effect. If that postulate is correct, then variations between our group and others in bacterial or mouse strains used for these assays may explain the apparent discordance in the *in vivo* results. A second reason for further analyzing the influence of BclA on the *B. anthracis* spore surface is the possibility that the hydrophobicity of BclA may dictate how spores initially interact with inanimate surfaces. If this theory proves to be the case, then evaluation of chemical ways to interfere with or modify BclA may lead to more efficient methods of surface sanitation in cases of an inadvertent or deliberate release of spores as well as sterilization of equipment in the laboratory environment.

Chapter Four

Discussion

Summary of results in the context of the specific aims

The first aim of this project was to clone, express, and purify BclA and assess its immunogenic properties. For those purposes, we designed primers and used them to amplify *bclA* from the Sterne strain of *B. anthracis*. We then cloned *bclA* into a vector that directed the incorporation of an additional nucleotide sequence for 6-histidines at the 5' end of the gene. Next, we expressed the N-terminally His-tagged version of the rBclA protein in *E. coli* and purified the recombinant protein by Fast Pressure Liquid Chromatography on a nickel affinity column. The resultant rBclA was approximately 70kd, a size consistent with the findings of Steichen et al. (109). The purified rBclA elicited specific antibodies in A/J mice when administered with the adjuvant Titer Max, but this immune response was not sufficient to protect the animals from challenge with 10 LD_{50} of Sterne strain spores. However, the anti-rBclA antibodies did slightly, but statistically significantly, prolong the mean time to death of the infected mice compared to controls inoculated with normal rabbit serum IgG.

The second aim of this study was to generate polyclonal antibodies against rBclA and to evaluate the capacity of these sera to block spore-related activity *in vitro*. To achieve these goals and in collaboration with the Naval Medical Research Center, we used rBclA (prepared as above) with an adjuvant to repeatedly immunize two rabbits over a period of 18 months. The final bleeds of these polyclonal anti rBclA sera were then purified to obtain IgG. Each of the resultant polyclonal anti-rBclA rabbit IgGs were of high titer (~ 200,000) as determined by ELISA. Additionally, these antibodies were

very specific for rBclA; in fact, that did not cross react with any of the other eight recombinant spore proteins generated by others in our laboratory. The rBclA IgG enhanced opsonophagocytosis of spores by mouse macrophages while concurrently decreasing intracellular spore germination.

The third aim of this investigation was to evaluate rBclA as a protective immunogen and to assess the capacity of rBclA antibodies to prevent anthrax disease in a mouse model. To fulfill this objective, I tried different immunization schemes that involved both rBclA and rPA. I found that rBclA enhanced protection conferred by sub-optimal immunization with rPA when given two weeks after immunization. The supplementary protection provided by boosting rPA-primed mice with rBclA correlated with the capacity of active immunization with rBclA or passive administration of anti-rBclA rabbit IgG to prolong the MTD of spore-challenged mice. In support of the possible use of this spore-surface- based antigen as a boost for PA-based anti-anthrax vaccine for humans, I detected little or no cross reactivity between rBclA and human collagen types I, III and V.

The fourth specific aim of my dissertation research was to assess the effect of BclA on spore germination, interaction with extracellular matrix proteins, and hydrophobicity. To achieve this goal, our collaborator, Dr. Scott Stibitz, constructed a *bclA* deletion mutant of *B*. anthracis Sterne strain 34F2, and I then characterized the mutant. First, I tested spore surface protein extracts of the wild-type and 34F2-Δ*bclA* spores for the presence or absence of BclA by western blot analysis. I detected no BclA band in the SSPE of 34F2-Δ*bclA*. Next, I compared the growth and sporulation capacities of the wild-type 34F2 with 34F2-Δ*bclA*. I found that the strains grew with

comparable kinetics and to indistinguishable levels. To evaluate the sporulation capacity of the wild type and mutant strains, I assessed spore formation over time in broth culture by both optical density measurement at 600 nm and phase contrast microscopy. Results by both sporulation assessment methods were similar for the wild-type and mutant strains.

The influence of BclA on spore germination rate, virulence, adhesion to extracellular matrix proteins, and hydrophobicity were assessed next. I found that the mutant spores germinated at a faster rate *in vitro* than did wild-type spores. When I compared the relative LD_{50}s of mutant and wild-type spores by intranasal or subcutaneous routes of administration, no statistically significant differences between the 34F2 and 34F2-$\Delta bclA$ were noted. However, the LD_{50} values I obtained were always lower for the mutant strain. Additionally, the MTD of mice given a 10^6 dose of wild type 34F2 spores was longer for the intranasal and subcutaneous routes compared to mice that received 34F2-$\Delta bclA$ spores. I then examined the binding of fibronectin and laminin to wild-type and *bclA* mutant spores and found that the interaction of both fibronectin and laminin with 34F2-$\Delta bclA$ spores was greater than with 34F2 spores. I also observed that that the hydrophobicity of wild type spores was greater than that of $\Delta bclA$ spores using two different hydrocarbons. Finally, I tested the outcome of heat treatment alone on spore survival over time. No differences were noted in relative viability of wild-type and mutant spores, but the mutant spores became remarkably more hydrophobic in the presence of heat. Taken together, the results of the fourth aim illustrate the contribution of BclA to the water repellent nature of the spore, the kinetics of spore germination *in*

vitro, and perhaps *in vivo*, as well as the extent to which spores can stick to host matrix proteins.

Discussion of Selected Topics from Chapters

<u>Recombinant BclA versus the native BclA glycoprotein</u>

BclA was first isolated and described by Sylvestere et al in 2002 (111). Since that discovery, BclA has been the subject of considerable interest because of its location on the outermost surface of the spore and, by extension, its presumed capacity to interact with host cells. Although we successfully cloned and expressed rBclA and characterized its immunogenic properties and function of this protein in this project, one possible criticism of our results is that we conducted all our experiments with recombinant BclA expressed from *E. coli* rather than the native glycosylated BclA from *B. anthracis*. We concede that purified glycosylated BclA would have been the optimal antigen to use in these immunological studies. We also acknowledge that we attempted to isolate the material but were not successful (not shown). Nevertheless, we feel strongly that our findings with sera derived from animals immunized with rBclA expressed in *E. coli* are reflective of the interaction of antibodies with the native glycoprotein on spores for the following reasons. First, Steichen et al. 2003 (109), who reported the generation of a panel of monoclonal antibodies against whole irradiated Sterne spores, noted that many of these monoclonal antibodies reacted with both a 250Kd band from spore extracts (size of glycosylated BclA) as well as to an ~70Kd band (size of rBclA from *E. coli*). These investigators concluded that the anti-BclA monoclonal antibodies were directed against

the protein and not the carbohydrate moieties of the native BclA glycoprotein. Second, we found that polyclonal anti-rBclA IgG not only bound to spores in an ELISA but also promoted opsonophagocytosis of spores by macrophages, results that indicate that the antibodies made against the *E. coli*-expressed protein recognized the native glycoprotein on spores. Third, we observed that anti-rBclA IgG prolonged the MTD when given passively to animals prior to spore inoculation even though the antibodies were not elicited using the native BclA on spores.

In spite of our contention that anti- rBclA antibodies can augment PA-based immunity to anthrax, we realize that the use of native glysolsated BclA expressed *from B. anthracis* would be preferable as a vaccine candidate for at least three reasons. First, several *E.coli* proteins are often co-purified with rBclA. Second, endotoxin is present in the preparations of rBclA used to immunize animals because that protein is expressed in *E. coli*. Third, the host might develop an effective immune response at a lower dose of glycoslyated BclA than is required for rBclA and this decreased antigen load might permit simultaneous administration of glycosylated BclA and rPA. We conclude that to conduct further experiments in higher animal models with BclA either will necessitate a switch to glycosylated BclA or, if that proves too difficult an undertaking, a decrease in the level of protein and endotoxin contaminants in the rBclA preparations used for immunization.

<u>*In vitro* assessment of polyclonal anti rBclA antibody</u>

The polyclonal anti rBclA IgG that we generated for this study not only recognized rBclA, spore surface protein extracts, and intact spores in ELISA but was also very high titered and did not cross react with other purified recombinant protein constituents of the exosporium. Furthermore, our mouse macrophage experiments revealed that the anti-rBclA antibodies not only enhanced phagocytosis of Sterne- strain spores but also reduced germination of the spores within these phagocytes. These findings are very similar to a recent report that an anti-spore antibody that recognized BclA, in addition to other spore proteins, enhanced uptake and reduced intra-macrophage germination (42).

That anti-rBclA IgG stimulates osponophagocytosis of anthrax spores by murine macrophages is perhaps not surprising. One can envision that the Fab portion of the antibodies might bind to the protein components on native BclA and coat the spores in such a manner that the Fc portion of those antibodies is available to engage the Fc receptors on the surface of macrophages. However, the mechanism by which these antibodies reduce germination of spores within the macrophage is not as readily apparent, but several explanations are plausible. First, and possibly most likely, the presence of antibodies that coat the spore surface may in some way stearically hinder access of germinants to germinant receptors on the spores. A second possibility is as the spores

begin to germinate the budding vegetative cells become mired in anti-rBclA/ BclA complexes. A third scenario that seems less likely is that the anti-rBclA antibodies are microbicidal for the vegetative cells in the environment of the macrophage. Whatever the mechanism of anti-rBclA inhibition of spore germination within macrophages, the opsonization of spores by anti-rBclA antibodies appears to influence the outcome of the interaction of spores with macrophage and favors the phagocytosis of the spore.

Studies of rBclA and anti rBclA antibody *in vivo*

To demonstrate augmentation of protection afforded by rPA, I first immunized groups of mice with different concentrations of rPA and challenged with spores to determine that a single 50 ng dose of PA protected approximately 50% of the mice. I then immunized mice with 50 ng rPA (the suboptimal dose) and 10 ug rBclA. When given with rBclA, rPA immunized mice did not elicit the same level of anti-PA antibodies as they did when rPA was given alone, and probably as a consequence, these dually-immunized mice were not protected from anthrax spore challenge. I then immunized mice with rPA and boosted 2 weeks later with rBclA. When this staggered immunization schedule was followed, the mice developed adequate antibody levels to rPA, as well as an antibody response to rBclA, and the animals were fully protected on spore challenge. I concluded that immunization with suboptimal amounts of the vegetative antigen rPA followed by a boost with the exosporium antigen rBclA leads to full protection of animals against 10 times the lethal dose of spores. The major concern with this approach is that it necessitates at least two immunizations. The ultimate goal of simultaneously administrating either rBclA and rPA or, as discussed above, glycosylated BclA and rPA will likely necessitate analyses of the kinetics and amplitude of the immune response to the individual immunogens given separately and together.

The reason that co-administration of 10 μg rBclA and 50 ng rPA (suboptimal amounts) was not protective and in fact reduced the anti-PA response in animals remains a subject of speculation. Our primary hypothesis is the difference in concentration of the two antigens was so great that simultaneous introduction of the two led to a focus of the immune response on the more concentrated immunogen. We did try to decrease the dose of rBclA from 10 μg to as low as 0.1 μg (we actually tried three doses: 10 μg, 1 μg, and 0.1 μg), but lower amounts of rBclA were not as effective as 10 μg when inoculated 2 weeks after rPA. Furthermore, none of the rBclA levels tested worked when given with rPA. We also experimented with administration of the two antigens at different sites in the animals but that also did not result in protection of the mice and still led to a diminished anti-rPA titer. Of note, when NMRI mice were vaccinated with plasmids that encoded rBclA and rPA, the animals were protected from Ames strain spore challenge (53). Therefore, with further experimentation, we remain optimistic that we can develop a regimen in which both antigens are given together. Such an investigation may not only require an alternate form of BclA but may also necessitate the use of a different animal model (see section on the A/J mouse model below)

The schedule for antibiotic therapy after *B. anthracis* spore exposure was designed to cover the possibility of a delay in the germination of inhaled spores. In fact, the current guidelines are for 60 days of treatment (5). Previously, Knudson and colleagues proposed that passive anti-PA antibody therapy in conjunction with antibiotic therapy may reduce the need for prolonged therapy. In this investigation, we hypothesized that administration of anti-rBclA IgG might reduce the extent of spore germination *in vivo* and the subsequent bacterial load (septicemia) with its ensuing

toxemia. With this concept in mind, I passively transferred anti- rBclA antibody to mice and challenged them with ten lethal doses of spores to assess protection, if any, afforded by the antibody. The mice showed prolonged survival when compared with normal rabbit serum IgG and PBS groups. This MTD data supports the utility of anti-rBclA antibody in prolonging survival even though the mechanism by which the antibody affords this survival benefit is clear.

Germination of $\Delta bclA$-34F2 spores

I found that the mutant spores germinated at a faster rate *in vitro* than did wild-type spores between 30 and 60 minutes of incubation in germination media and that the mutant spores germinated to a statistically significantly greater level than wild-type at 45 and 60 minutes of incubation. Conversely, Bozue *et al* reported no statistically significant differences in germination between the Ames strain and its *bclA* mutant. An explanation for this apparent discrepancy between their publication and ours may be that there are intrinsic differences in the kinetics of germination between Sterne and Ames strains. However, the Bozue *et al* publication, their mutant did appear to germinate to a somewhat greater extent and at a slightly faster rate than wild type, as evaluated by their spectrofluorometric assay for germination (16). In addition, another factor that may explain the differences between our finding and the conclusions drawn by Bozue and colleagues about germination of their BclA mutant spores is that there are different concentrations of BclA on the exosporium of Sterne and Ames strain spores (data not shown).

Our results also suggest that $\Delta bclA$-34F2 spores may germinate slightly faster than wild-type Sterne spores *in vivo*. When I compared the relative LD_{50}s of mutant and wild-type spores by intranasal or subcutaneous routes of administration, no statistically significant differences between the 34F2 and 34F2-$\Delta bclA$ were noted. However, the LD_{50} values we obtained were always lower for the mutant strain. Furthermore, the MTD of

mice given a 10^6 dose of wild-type 34F2 spores was 4.2 days for the intranasal route and 4.4 days for the subcutaneous route compared to 3 days for mice that received 34F2-$\Delta bclA$ spores by both the intranasal and subcutaneous routes. In aggregate, these findings imply that in the absence of BclA, spores may germinate within the host at a more accelerated rate.

A/J mouse/Sterne challenge model for vaccine studies:

Several different animal species, i.e. mice, rats, guinea pigs, rabbits, and monkeys, have been exploited as models of anthrax disease although these animals differ considerably in their natural resistance to infection. We elected to use the A/J mouse strain for our studies because these animals are particularly susceptible to the Sterne strain of *B. anthracis*. Indeed, Welkos and colleagues (126). reported that A/J and DBA/2J were the most sensitive of 10 inbred lines they tested by subcutaneous challenge with this non-capsulated, toxin-producing anthrax isolate; the LD50 values were ~ 1000-10,000 fold lower than for the remaining 8 inbred animal lines examined. Conversely, all the mouse strains evaluated by Welkos and collogues were killed by the fully virulent Vollum 1 B strain at doses below 50 organisms; however, the time to death (TTD) of the inbred lines after challenge with a single lethal dose of spores varied [fell into 3 groups: short (A/J and DBA/J), intermediate, or more delayed]. Since the Sterne strain of *B. anthracis* causes similar bacteriological and pathological signs in the final stages of disease as those observed with fully virulent strain (126), we contend that the combination of A/J mice with Sterne as a challenge strain is valid for conducting initial studies to assess vaccine efficacy. Moreover, the application of this particular host: bacterial isolate permutation in our investigation alleviated the need for a biosafety level 3 animal facility, higher challenge dose, vaccination of the investigators, and afforded us the opportunity to observe protection that might otherwise have not been so readily

evident. On the other hand, we acknowledge that the A/J strain of mice is deficient in C5 component of complement encoded by the Hc gene and is thus an immuno-compromised host (127). Therefore, as mentioned in the following section on future studies, we intend to validate our findings in a different animal model with a fully virulent *B. anthracis* challenge strain.

Future directions

This study provided a proof-of-concept of the benefit of spore proteins as additional immunogens in rPA based vaccine. There are several aspects of our investigation here that will require further analyses or alterations in the reagents or their applications. For example, in our immunization studies, we required a high dose of rBclA compared to rPA. Moreover, we could only demonstrate additional protection by rBclA over that of immunization with suboptimal doses of rPA alone when the immunizations were separated by a period of two weeks. Further dosage and scheduling studies are needed to determine the best immunization modality for these proteins for maximum efficacy as a vaccine. Furthermore, there are other exosporium-associated proteins that may be useful as additional components of a PA-based vaccine. The delineation of the right combination and exact dosing regime of each antigen may prove to be a daunting task, but perhaps a worthy endeavor. Additionally, even though we did not detect any cross reactivity between human collagens and BclA, consideration should be given to generating a rBclA protein truncated by 134 amino acids at the carboxy terminus of the molecule for future vaccine studies. This truncated protein would not contain the collagen like region (CLR) and thus should eliminate any concerns regarding autoimmune diseases triggered by vaccination. Lastly, the Sterne strain used in this study is attenuated in that it does not possess a capsule and the A/J mice used have a complement deficiency (126). Thus, these studies should be repeated with a fully virulent *B. anthracis* strain and a fully immuno-competent animal model.

When we initiated these studies, BclA was postulated to play a role in the interaction of spores with the host. Here we have clearly shown that BclA contributes to spore hydrophobicity and to the greater association of spores devoid of BclA with two extracellular matrix proteins. This latter finding suggests that one or more exosporium proteins that are normally masked by BclA may readily associate with ECMs. The identification of such specific proteins and an examination of the roles that they may play *in vivo* are additional areas of future research. Lastly, BclA may also function in spore survival in the soil microenvironment, a postulate that was not examined in this study but clearly warrants further investigation to enhance our understanding of the *B. anthracis* infection cycle in nature.

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